TECHNIQUE OF ANESTHETIZATION AND OF OVARIECTOMY IN THE RAT

W. H. SCHULTZ
Department of Pharmacology, University of Maryland

TWO FIGURES

INTRODUCTION

The use of the white rat for endocrine experiments in which surgical operations are involved has assumed so prominent a place in pharmacological investigations as to warrant an attempt at a standardization of the methods used. The items of technic offered at this time are born of experience and are put on record with a view to aiding workers entering this field of investigation.

The white rat, selectively bred and raised under carefully controlled conditions of diet and housing, is well suited to the requirements of modern endocrine technic. 'Body form' and 'rat reactions' are more important than 'body weight' as factors governing the selection of experimental material. Pedigrees and known physiological cycles in the rats' life history are most important to know if one is making comparative studies of glandular structure and secretory function. Hence there has been raised a colony of rats in our laboratory in which certain essential factors have been brought more or less under systematic control. (See 'General discussion' at the end of this article.)

ANESTHESIA

In this day of modern surgery, ether anesthesia is looked upon with such complacency that its side actions are either not recognized or ignored. The pharmacologist, however,

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with his interest centered in the reaction of living protoplasm toward chemicals, knows that different cell groups of an animal as well as different species of animals show a wide range of susceptibility to ether's toxic action. Duration as well as intensity of exposure to ether vapors are factors which determine whether or not the changes induced in certain cell groups shall be reversible or non-reversible.

It is the non-reversible ether reactions that lead to post-operative complications. The pathology resulting from too deep narcosis or too prolonged exposure to ether vapor augment both the death rate and the untoward action of the operation itself. A statistical study of results in this laboratory by Evans(8) of the influence of total adrenalectomy in white rats indicates that one method of anesthesia reduces the side actions of an ether adrenal operation to a minimum. Measured quantities of ether will aid greatly in preventing too rapid and too deep a narcosis, undue irritation with resulting inflammation, excitement, or secretion of various products. Primarily it must be borne in mind that a good anesthetist is after all an artist guided to success not merely by formulae, but also by an instinctive feeling of rightness born of a knowledge and understanding of the animal's reactions. The animal is placed within a jar, as illustrated in figure 1, until it has passed into a stage of depression. It can then be tied upon the operating board without inducing fear reactions. This stage of inducing anesthesia is included in stage I.[1]

Stage I. Presurgical

This period extends from the time of first ether inhalation up to that time in which all is in readiness for the skin incision. Arrange a fresh towel in a clean anesthetizing jar (fig. 1), place the rat upon the towel, close the lid, and shove the jar into its holder. Measure 3 cc. of ether into a sepa-

[1] The phrase 'stage of anesthesia' as used in this paper has not the same meaning as given in the standard text-books of pharmacology. 'Stage I' and 'stage II' are used for the sake of convenience of grouping degrees of narcosis best suited to the type of operative procedure described.

ratory funnel and insert the stem into the hole of the lid. The stopper of the funnel is removed, the tip of the stem raised against the median surface of the jar, and simultaneously with

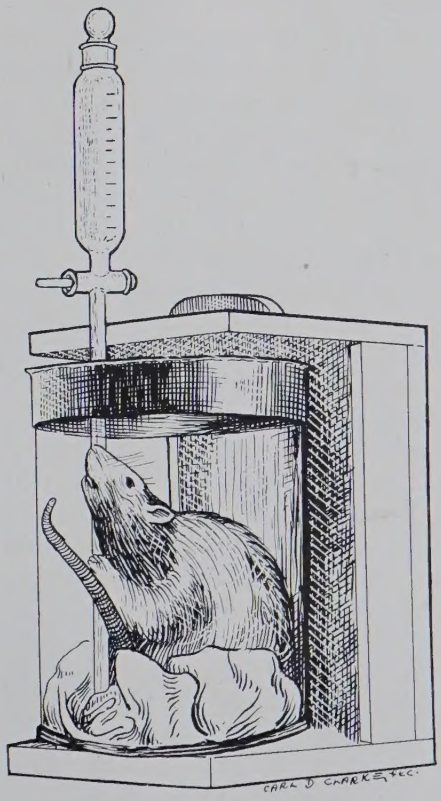

Fig. 1 Illustrates an outfit for anesthetizing rats, mice, and guinea-pigs. The glass receptacle is 15 cm. in diameter and 20 cm. high. The lid is provided with a 4-cm. flange which fits the jar snugly, thereby preventing rapid exchange of gases with the outside air. The volume of the container is sufficient to provide ample oxygen during the time necessary to anesthetize the animal. The lid is provided with one hole near the margin through which the stem of a 10-cc. separatory funnel passes next to the inner surface of the glass jar. The frame is made of $\frac{1}{2}$-inch white pine constructed so as to provide a convenient means of carrying the rat jars or stacking vertically or horizontally. The lid is always held securely in place and the jars when stacked horizontally make excellent sets for observing treated rats.

the opening of the stopcock, the outflowing ether is spread over the glass surface by moving the stem tip through as wide an arc as possible. This insures rapid evaporation. The heavy ether vapor concentrates in the lower portions of the jar, but through its vapor pressure, saturates the region above the towel to about the right anesthetizing point. The rat through its olfactory sense detects this condition and stands upon its hind legs in an attempt to avoid the irritant ether. This reaction results in a more gradual inhalation of ether and automatically induces the type of anesthesia most nearly desirable.

Soon the rat loses control of its hind legs and slumps upon the towel. At this point, if the animal's head drops within the range of the densest ether vapor, almost pure ether vapor may be inhaled. The animal respiring at a rate of 80 per minute may suddenly stop breathing and die. Hence care should be taken that the head rests upon a ridge of the upturned folds of the towel. About one minute following the initiation of this stage of depression, the muscles of the neck and back lose their tonus and the animal becomes limp. The breathing is regular, with a reduced but efficient exchange of tidal air and a rate of about 90 to 120 respirations per minute. It is at this stage of anesthesia (about three minutes following the running in of the ether) that the animal is removed to the operating board and tied. From now on the ether is administered from the ether bottle (fig. 2).

Figure 2 illustrates the animal tied upon the operating board ready for the ether-bottle method of anesthetization. It is customary to give additional ether only when the animal shows signs of struggling; frequently this does not occur until all of 'Stage I. Preparation' under 'Operative procedure' has been completed. Light anesthesia is thereby maintained until ready for the second stage in the operative procedure (p. 107).

As soon, however, as the anesthesia becomes too light and the motor reflexes return, 2 cc. of ether are run upon the cotton of the anesthetizing bottle (fig. 2, *2*) and the nose of the

Fig. 2 Illustrating: 1. Method of tying the animal upon the operating board. 2. The use of the ether bottle. 3. Size and location of the skin incision and its deflection. 4. Size and location of the dorsolumbar incision made by spreading the tips of the tweezers. 5. Location of the transverse iliac-crest line shown by a thread stretched over the skin painted with tincture of iodine.

rat is shoved into the mouth of the bottle. The initial anesthetizing action is roughly maintained by dropping fresh ether upon the cotton every few minutes, and a method of gaging the proper amount of available vapor is to test its olfactory pungence.

With a given ether concentration within the bottle, it is soon learned at what distance the rat's nose should be held from the cotton of the ether bottle. Deep surgical anesthesia is established within one to two minutes by thrusting the rat's nose within the ether bottle close to the bottom. The cone-like muzzle of the rat nearly closes the mouth of the bottle, the warm expired air vaporizes the ether, and nearly saturated vapor is inhaled. Hence respiratory movements and muscle tonus, especially of the abdominal muscles, must be closely watched. The margin of safety left after the relaxation of the abdominal muscles is small, since the respiratory center reflexes drop out soon after abdominal relaxation and with but a slight increase of ether in the blood. The proper degree of surgical anesthesia for stage II of the operative procedure is assured by the presence of the following reactions:

1. There will be regular even respiratory movements with no trace of tremor-like twitchings of the diaphragm or intercostal muscles and a rate of respiration of about 84 to 94 per minute. (A rate below 80 is undesirable and is fraught with the danger of sudden respiratory failure. Indeed, when the inhaled ether vapor is concentrated, rats may suddenly die, even though the respiratory rate is 80 and the tidal air volume apparently large.)
2. There will be a trace of the palpebral reflex if the eyes have been protected from direct contact with ether vapor. Pinching of the tail, or a toe of the hind foot, will not elicit the jerk reflex.
3. There will be a lack of both skin and jerk reflexes ordinarily elicited by pinching the skin over the mediodorsolateral area by means of a Halstead's forceps, and the same reflexes will be absent even when cutting through the skin itself.

Stage II. Postoperative anesthesia, including the period of recovery

As soon as possible, ether inhalation should be reduced to a minimum. Dressing of the wound, releasing ligatures,

applying number tags, and preparing the animal for the convalescent cage should be done under very light anesthesia.

Very light anesthesia (starting with the early part of stage I, or following removal of the ether until this stage is reached) may be established and maintained for some time by placing the ether bottle so that its rim is near the tip of the nose of the animal. In this stage also, the degree of anesthesia can readily be estimated by testing, with a bristle of thin broom straw, the sharpness of the palpebral reflex. When the narcosis is still lighter, bordering upon the stage of sleep, simple touching of the incisor teeth with the rim of the ether bottle will elicit the gnawing reflex. One can, through this reflex, after some practice, estimate the depth of this stage by the degree of intensity with which the rat nibbles or bites the glass rim. There is a rather close relation existing between the animal's increasingly purposeful type of biting and the return of the postural reflexes as evidenced by struggling to free itself. Of course, no pain stimuli are allowable at this stage of anesthesia. Dressing of the wound, applying of number tags, and even release of the leg strings should have been completed by the time the animal begins to struggle.

OPERATIVE PROCEDURE

Stage I. Preparation

Anesthetize the rat as outlined under anesthesia, stage I. Then gently stretch the animal upon an appropriate operating board.[2] The legs should be spread sufficiently taut to prevent the rat rotating its head under its fore limbs, but not tight

[2] A very convenient type of board can be improvised by stretching a double layer of an old towel over a ¾-inch pine board, 18 × 30 cm., and driving an inch box nail in each corner of the board about 3 cm. from its edge. Slant the brads so that the head points toward the corner of the board, making an acute angle of 15° to 20° with the surface of the board. Use a double strand of 3-ply cotton cord 20 cm. long looped up at one end. Such a cord is used as tying strings, which with a double turn on the nails, grips readily in the acute angle. Better still is the Schultz rat board described in the Journal of Laboratory and Clinical Medicine, 1932.

enough to interfere materially with its respiratory movements (fig. 2).

With a pair of clippers[3](1) remove the hair from a rectangular area of the skin, beginning from 5 mm. caudad to the intercrest line[4] and extending dorsad to about the first lumbar vertebra. The pressure of the clippers necessary to cut the hair short will so flatten the skin that the swath will give the proper width of the rectangle for each type of animal used.

If, as is preferred by some operators, it is desired to shave the median portion of this area, lather it with "Sapo Mollis" and shave with a new Gillette razor blade. The blade is held by some form of handle adapted to surgery(2) or the hair can be removed chemically.[5] This area is then cleansed and dis-

[3] For operations of the type described in the text the instruments cited are worth trying. In order to be specific, the twenty-third edition of Kny-Scheerer's catalog of surgical instruments has been used for citing the numbers herein listed.

1) B/1985. Mundus hair clippers, ½ mm. cut.
2) B/1960. Handle for holding Gillette razor blades.
3) A/2052. Dissecting forceps, half-curved, 12 cm.
4) A/2050. Dissecting forceps, half-curved, fine, 12 cm.
5) A/2459. McClure's microscopic scissors with spring, straight.
6) A/ 573. Halstead's mosquito forceps.
7) B/7841. Mayo's scissors, 12 cm.
8) B/1118. Horsley's forceps.

[4] The intercrest line is the line passing through the right and left iliac crests at the point of juncture of each respective lateral ridge with that of the crest. This line is chosen as a reference coordinate because it can easily be approximated through grasping between the index finger and the thumb, the lumbar vertebra, and their associated dorsolateral muscles. Then with a gliding caudad movement the position of the projecting iliac crest on either side can readily be felt. Through palpation the crest points are determined and an imaginary line drawn through them (see item 5 in the legend to fig. 2). In the dissected animal this can be done accurately. In practice, however, in this particular surgical technic, the position of the line, used as a point of reference, is determined after gently stretching the tail of the anesthetized rat (tied as per directions) so that all vertebrae are practically in a straight line. The iliac crests under this condition assume a standardized position. The middorsal line will be found defined in Milner's "Comparative Anatomy."

[5] Mix two parts of sodium sulphide and one part plaster of Paris with just enough water to make a thin paste. This paste is rubbed into the hair thoroughly with a cotton swab or fiber brush, then scraped off along with the hair by means of a blunt instrument. Barium or mercury sulphide is a better solvent, but when licked off by the animal, both of these are more toxic.

infected. If the U.S.P. X tincture of iodine is used, it must be freshly made and applied liberally.

Stage II. Incisions of the skin and abdominal wall and major surgical operations

Pinch between the jaws of a pair of tweezers(3) a fold of the skin at the intersection of the middorsal line and the intercrest line. With a sharp pair of scissors snip an opening through the hide, grasp the caudad margin of the opening with the tweezers, drawing the hide caudad until taut, insert the pointed tip of the scissors, and cut straight forward to a distance of about 20 to 30 mm. (fig. 2).

Beginning on the right-hand side, grasp the cut margin of the skin with a Halstead's forceps about 15 mm. dorsad from the starting point of the incision. The hide is then freed from its areolar tissue moorings over a semicircle, the radius of which is about 10 to 15 mm., rotated about the tip of the grasping hemostat as its center. The white bands of the lumbodorsal fascia, the dorsolateral surface of the m. longissimus dorsi, the white shadow of the lumborenal fat mass, at times the upper portion of the uterine horn fat, and the faint outlines of portions of the kidneys and the intestines come into view. Locate more accurately the outlines of the cream-colored band of lumborenal fat.[6] Starting at its lowermost distal margin near the crest line of the ilium, follow it cranialward for one-third of its length (about 10 mm. in a 150-gram rat). A few millimeters distal to the fat margin will be observed a small fusiform area. Over this area, snip with a pair of scissors the connective tissue down to the red fibers of the m. obliquus abdominis externus. Push the curved tips of a pointed pair of half-curved forceps(4) between the muscle bands through the peritoneal membrane into the abdominal cavity (3 to 5 mm.); then spread the jaws of the forceps, thereby stripping apart the muscle fibers, forming

[6] In the lean and poorly nourished animals the outline of a portion of the uterine horn and the orange yellow of the ovaries can be seen through the dorsolateral walls.

an oblong hole about 5 to 7 mm. long. If done properly there will be no bleeding. With the jaws of the tweezers still in the opening, allow them to spread open a few millimeters, then reach deeper into the abdominal cavity, finally turning slightly toward the middorsal region and a little dorsalward in the region of the angle made by the kidney and vena cava; grasp the delicate uterine fat and carefully work it through the opening. The characteristic color of the ovary readily differentiates it from the fat mass. Having located the ovary to be removed, proceed by one of the three methods outlined below.

Technic no. 1. Removal of the germinal tissue, leaving the fallopian tube intact except where the periovarian membrane is cut. The ovary is drawn through the abdominal incision and spread upon a sterile glass slide. It is strongly illuminated by a beam of light from a dissecting microscope lamp and a low-power microscope is focused upon the periovarian membrane and ovary. A fine silk ligature is tied around the ovarian artery including a portion of the mesosalpinx attachment to the ligamentum latum just distal to the hilus of the ovary. A second fine ligature is passed around the structures intimately associated with the periovarian membrane just distal to the fallopian tube papilla opening into the periovarian sac. The periovarian membrane is now cut open with microscissors(5) and the ovary extruded. Having located the ovarian pedicle, sever it, if possible, with one snip of the microscissors. When it is impossible to do this without bleeding, then use the second ligature to tie off all anastomosing branches of the arteria uterina. The ovary along with part of the periovarian membrane and ovarian fat body are excised. The most distal ligature attached to the round ligament and the ligature tied near the distal end of the fallopian tube are now drawn close together and tied to each other. This aids in holding the fallopian tube in its original position after removal of the ovarian or germinal tissue proper. If done properly there will be no bleeding. There should also be left

behind no germinal tissue from which ovaries can later develop. Sometimes in grasping the ovary with the tweezers, the cortex and medulla are crushed and unless due care is exercised, germinal tissue may thus be liberated. Likewise small particles of this same tissue may be set free when more than one cut with the scissors must be made. This, of course, may defeat the object of the operation, since there remains the possibility of the regeneration of the ovary. Any physiological reactions indicating this possibility will eventually have to be studied and controlled by a final postmortem study. Histological sections and microscopic examinations of the tissue involved will have to be used to decide whether ovarian tissue is present or not.

Technic no. 2. Removal of the ovary, the periovarian membrane, and the fallopian tube. In this operation the procedure is the same as that described in technic no. 1 up to the point of tying the ligatures. One ligature is tied distal to the periovarian membrane close to the attachment of the mesosalpinx to the ligamentum latum, including the arteria ovaria. A second ligature is tied around the fallopian tube so as not to include the papilla of the uterine horn. This leaves but a trace of the fallopian tube in situ. Of course, the uterine horn blood vessels are included or a third ligature is tied around them. The entire periovarian membrane, ovary, and all of the fallopian tube distal to the second ligature are excised. The first and second ligatures are then drawn together and tied. This procedure holds the uterine horn in place until scar tissue anchors it permanently. Postmortem findings have demonstrated that long after such operations have been made the uterine horns lie in approximately their normal positions.

Technic no. 3. Removal of the ovary, periovarian membrane, fallopian tube, and distal portion of the uterine horn. This operation is the simplest of the three types. The ovarian fat body is drawn through the dorsolumbar incision and along with it the periovarian membrane with its included ovary, the fallopian tube, and about 1 cm. of the uterine horn. The

ovarian mass is grasped with a pair of tweezers so that it forms the top of an inverted 'U.' One side of the 'U' is made up of the structures dorsad to the ovary, the other by the coiled fallopian tube and a portion of the uterine horn. One ligature is now so placed around the folded tissues on the lower portion of the 'U' that when tied, the uterine horn and the structures caudad to the ovary are drawn together. With a pair of microscissors the ovary, periovarian membrane, fallopian tube, and about 5 mm. of the uterine horn are excised. There is no bleeding. The remaining uterine horn is anchored to the structures originally dorsad to the hilus of the ovary. When not interested in the problems restricted to the removal of germinal tissue alone, this type of ovariectomy is simple and effective. It is well adapted for testing certain phases of the action of so-called sex hormones. When properly done, it insures total excision of the germinal tissue. This we have demonstrated conclusively by physiological tests and by postmortem studies of the degenerated sex organs of rats.

With the completion of any one of the above-described operations, the residual parts are carefully tucked back into their normal position. The margins of the opening through the muscles are drawn together, the fascia replaced over the cleft, and the cut margins of the hide are brought into juxtaposition. With the back of a half-curved pair of forceps, the right and left skin margins are so pinched together that the parallel cut edges are exposed upward. One—sometimes two—7/16-inch Michel's suture clips are set in place. The wound is then thoroughly painted with tincture of iodine and dressed with flexible surgical collodion.

The animal is now placed in a convalescent cage upon a clean towel until it recovers completely from the effects of the ether. It is then finally transferred to a group cage for final observation and study.

GENERAL DISCUSSION

1. Rat histories accompanied by selective breeding

Through careful selection the rats have been bred and pedigrees kept through at least ten generations before entering upon a special endocrine investigation. Dominant and recessive types thus become evident and can be taken into account. Organ structure and function corresponding with these types present themselves for study, and already some of the differences found in the unassorted stock animals, sometimes referred to as idiosyncrasies, at other times passed over as unavoidable differences, can be either eliminated or explained. Under such conditions, data on body weight correlated with age and body form acquire a new meaning.

2. The importance of diet in producing normal female sex organs for this type of experiment and for securing maximum responses to test substances

In 1910, two series of experiments were planned and started. In one series white mice were used. The stock food was the standard cake or a slight modification of it, used by Ehrlich in his toxicity experiments with arsenic compounds. Water ad libitum and a limited amount of whole cow's milk were part of the daily ration. The toxic substances were purine compounds, most prominent among them being caffein. In the second series, guinea-pigs were used. The stock food was rolled oats, a limited amount of whole cow's milk and water. Excelsior packing material was used to bed the cages. These animals were sensitized by injecting crystallized egg white or some other protein that might later be included in the experimental diet, the idea being to study some of the problems of so-called food sensitization of the anaphylactic type.

In each of these experiments complications set in, since the twenty- to thirty-day periods brought out reactions in the control animals now known to be associated with deficient diets. These are extreme cases, but they illustrate how intent pharmacologists were at that time upon simplifying the diet

to avoid antagonisms between the unknown constituents of a complex diet and the drugs used in the toxicity experiments. As might be expected, important facts were masked in the short-time experiments when potent drugs were used. But when slowly acting drugs were used, the control animals emphasized the influence of the deficient diet. As a result of these experiments I have always avoided the use of special diets with which control animals could not be kept in a normal condition of growth and physiological sensitivity over long periods of time. The importance of this idea was still more emphasized when mice, rat, guinea-pig, and chicken colonies were developed from the standpoint of thoroughbreds; new and additional knowledge of diets was gleaned first-hand and from the experience of other workers. There is certainly no more convincing method of emphasizing this kind of knowledge to pharmacologists and endocrinologists than to develop colonies of thoroughbred animals either from the standpoint of prize winners or from the standpoint of producing standardized animals for experimental biology.

In raising and controlling a rat colony the high price of cow's butter introduces an economic problem and we attempted to solve it by using margarines. It was, however, soon evident that the new series of uterine and ovarian tests did not duplicate those made with rats fed upon cow's butter. The vaginal smears ceased to run true to form and the contractions of the uterine horns diminished in intensity and regularity. Even when the amount of margarine fats in the same stock diets was increased two- to three-fold that of the butter fat, there was still evidence of a deficiency of essential vitamins. A. Scheunert reported typical deficiency syndromes with margarines, both from vegetable and animal fats, but this deficiency was more pronounced with the margarines from vegetable fats.

Usually the object of an ovariectomy is to use the animal as a test object for some group of physiological reactions in which the sex organs are involved. With our present knowledge of the interrelationships existing between the endocrine

glands, only standardized animals should be used. In emphasizing the importance of this fact one cannot ignore the valuable contributions of the following investigators: Evans and Scott(1), Evans and Bishop(2), Evans and Burr(3), Mattill and Conklin(4), Mattill and Clayton(5), Reynolds and Macomber(6), Sure(7), and their contemporaries whose articles will be found in cross references cited by these respective writers.

This literature covering the past ten-year period represents entirely new ideas on the specificity of food substances. Dietary science has advanced to such a stage of development that one can predetermine what group reaction will ensue. A combination of foods can be chosen so that any of the sterility factors or a combination of them may obtain. For example, the following conditions may be induced at will by a proper choice of diet:

1. The normal sex cycles, as evidenced by the type of vaginal smear, can be rendered atypical.
2. The uterine horns can be so altered that either implantation of the ova does not take place, or if it does, then atypical embryos result.
3. Apparently normal ova or normal sperm may obtain, but fertilization does not follow mating.
4. Fertilization may ensue, but no young are born.
5. Birth of young may result, but the offspring usually die early.
6. The first generation of offspring may reach maturity, but prove to be sterile.

If such a wide range of reactions toward diets which otherwise seemed to support life and maintain body weight can be obtained at will, experiments designed to throw light upon the function of the different units of the sex apparatus must be standardized and the diet must be complete. Or when a diet is rendered deficient for experimental reasons, care should be taken to state accurately the type and amount of food consumed.

SUMMARY

1. A method of inducing ether anesthesia in the rat is described. The method involves a minimum of physiological disturbance resulting from excitement in handling and irritation by the anesthetic.

2. The cardinal reactions of the rat while under light ether anesthesia, suitable for all pre- and postoperative procedures, are outlined in terms of somatic neural manifestations.

3. The cardinal reactions of the rat while under a depth of ether narcosis best suited for major surgical operations are outlined.

4. Emphasis is laid upon the importance of brevity of ether narcosis, for light anesthesia by virtue of ether's irritant action, and for deep surgical anesthesia from the standpoint of non-reversible reactions.

5. A method for performing ovariectomies is described for the rat. Three types of technic are outlined. Technic 1 is a method for removing the germinal tissue, leaving the fallopian tube intact except where the periovarian membrane is cut. Technic 2 is a method for removing the ovary, periovarian membrane, and fallopian tube. Technic 3 is a method for removing the ovary, periovarian membrane, fallopian tube, and the distal portion of the uterine horn.

6. Since ovariectomies in experimental biology are frequently made with a view to studying the reaction of the residual sex organs and disturbances in endocrine physiology, a brief discussion is introduced on the importance of standardized animals and the importance of pedigreed stock raised upon diets of known composition.

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THE PRENATAL GROWTH OF THE ALBINO RAT [1]

A. W. ANGULO y GONZÁLEZ
The Wistar Institute of Anatomy and Biology

SIX CHARTS

During the course of my research upon the anatomical interpretation of the development of behavior in albino rat fetuses, it was found necessary to study the fetal development of this species. The work suggested itself as the result of an attempt to interpret certain discrepancies found in the behavior reaction of some of the litters.[2] It was noticed that sometimes fetuses of litters of the same age did not react alike; also, that within the same litter fetuses differed in their behavior reaction. Thus, it was thought that a possible explanation of these differences might be found by the study of their prenatal growth.

Search of literature reveals that a great deal of work has been done upon prenatal growth in mammals: Lowrey ('11), Stotsenburg ('15), Draper ('20), Hartman ('28), Ibsen ('28), Warwick ('28), Latimer and Aikman ('31); and in man: Jackson ('09), Streeter ('20), Scammon ('27), and Scammon and Calkins ('29). However, it seems to me that often important factors which affect considerably the growth of the fetuses have been disregarded in these studies. King ('15), in her work on the weight of the rat at birth and the factors

[1] I wish to express to Dr. H. H. Donaldson my appreciation of his valuable help and criticism in the presentation of these data, also to thank him for putting at my disposal the service of his assistants, Miss Mary E. Frings and, later, Miss Beatrice Roberts. To them I am indebted for the weighing of the material presented in this contribution.

[2] In the early part of my work the female rats were chosen at random, disregarding age and other important factors that since have been found to affect the growth of the fetuses.

that influence it, has shown that the weight of the rat at birth is influenced by the following factors: age, physical condition, and body weight of the mother; size of the litter; position of the litter in the litter series, and the length of the gestation period.

This work is presented with the hope of showing that if these factors enumerated by King are taken into consideration in the selection of material, the age of the fetuses can be correlated with their growth in length and weight, and the correlation of their age with their physiological development is facilitated. Thus, the albino rats used for my experiments were selected with these considerations in mind.

In this presentation, emphasis is placed on the development of fetuses under standard conditions. Considerable material has been collected which shows the effect of deviation from these standards. However, in order to preserve the clarity of the graphs showing the normal development, only a few typical cases of deviation from these standards have been introduced.

AGE OF THE MOTHER

Long and Evans ('22) have shown that the female albino rat reaches sexual maturity (rupture of the vaginal membrane) at about 72 days of age, also that the first ovulation takes place at about 77 days of age. King ('15) states that the albino rat usually casts her first litter at from 90 to 120 days of age. Greenman and Duhring ('31), however, report birth in albino rats as early as 55 to 57 days of age. They also state that when mating occurs previous to 110 to 120 days of age, the litter cast is often unsatisfactory. Therefore, for my work, first litters from female albino rats of 110 to 190 days were selected. The distribution of the females used is given, according to age groups, in chart 1.

Since the age of the mother rat affects the size of the litter and the weight of the young (Slonaker, '12, and King, '15), it was thought advisable to determine whether the range of ages chosen for this work was the most desirable; in other

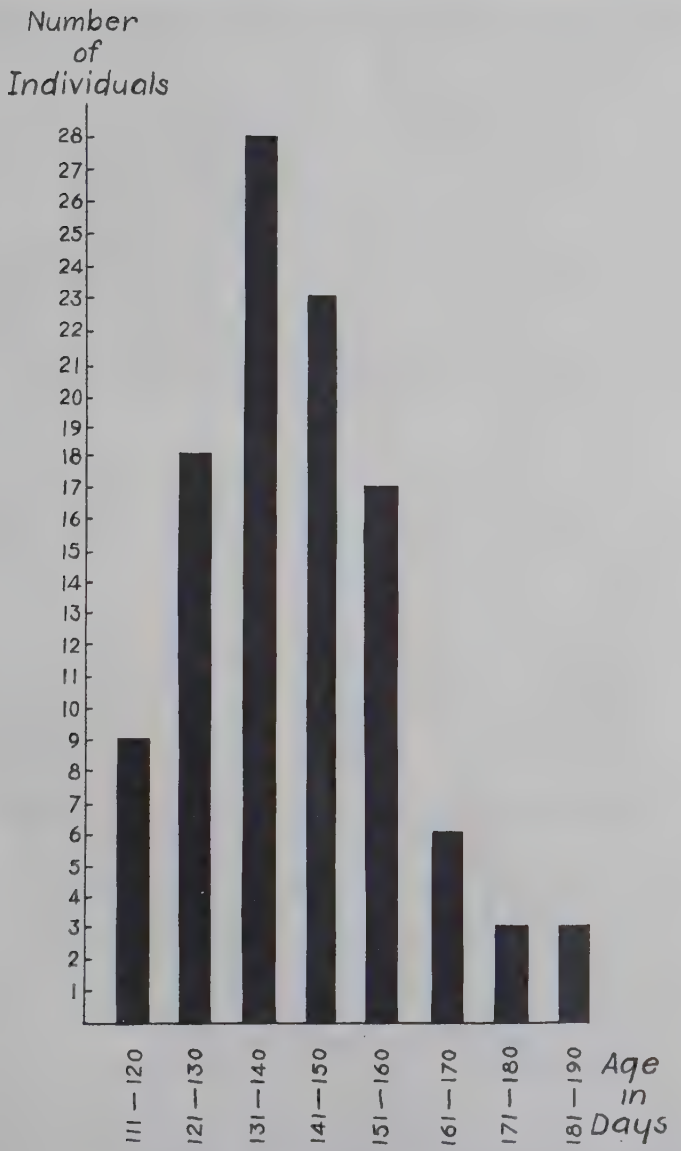

Chart 1 Shows the distribution of females according to age group at the time of the first litter.

words, whether within this range of age the female rats were at their optimum physical and reproductive condition. This was measured by means of the degree of fecundity of the mother. The results of these observations are shown in table 1. According to this table, the rat has its largest litter between 110 to 130 days of age. After this period the litter decreases in size. The larger averages found for 171–180 and 181–190 may be due to selectivity by reason of the small number of cases (three). The lowest average (eight) found in this determination is considered to be the most frequent litter size for normal rats (chart 2). From this we can infer that the rats used for this determination were in good physical and reproductive condition.

TABLE 1

Average size of the litter according to the age of the mother

AGE OF MOTHER	NUMBER OF LITTERS	NUMBER OF FETUSES	AVERAGE NUMBER OF FETUSES PER LITTER
110–120	9	90	10
121–130	21	214	10
131–140	35	321	9
141–150	24	223	9
151–160	16	128	8
161–170	5	41	8
171–180	3	30	10
181–190	3	27	9

DISTRIBUTION OF FETUSES ACCORDING TO SIDE AND POSITION OF THEIR HEADS

Observation was made as to the number of fetuses found in each uterine horn; also, the orientation of the fetuses within the horns was determined. Those with their heads toward the vagina were marked as 'head down,' and those with their heads away from the vagina as 'head up.' Table 2 shows the result of this observation.

According to side, there is a very small difference (4.5 per cent) in favor of the left horn. Thus there is almost equality of distribution of the fetuses between the two horns. This result substantiates the findings of Stotsenburg ('15) for the

albino rat, Draper ('20) and Ibsen ('28) for the guinea-pig, and Warwick ('28) for swine.

The egg cylinder (seven to eight days after insemination) is oriented with its long axis perpendicular to that of the uterine horn (Huber, '15). This arrangement is maintained until ten days after insemination (Nicholas, '30). From this

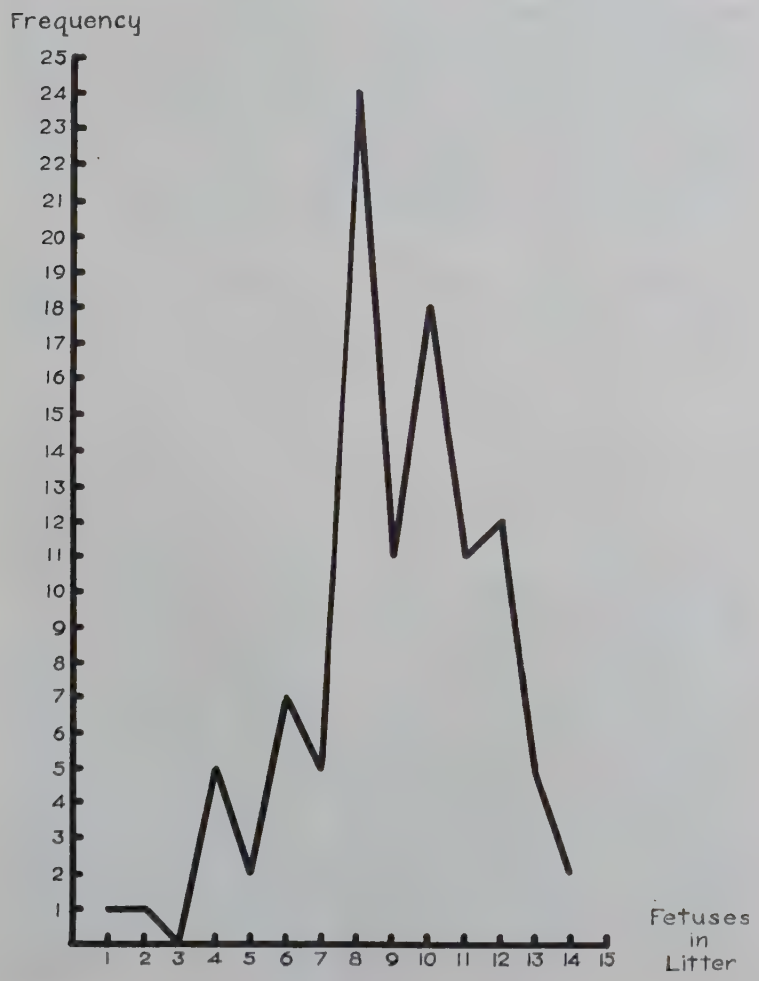

Chart 2 Shows the frequency of litter size.

age to thirteen days after insemination there is no definite orientation of the fetuses. However, after this age the fetuses orient themselves with their long axis parallel to that of the uterine horn, unless crowding prevents them from doing so.

According to my record, the number of fetuses with head down is nearly equal to that for those with head up. This observation disagrees with that of Ibsen, who finds in the guinea-pig a difference of about 80 per cent in favor of fetuses with head down. Ibsen is of the opinion that fetuses near the vagina have less space in which to turn, and are therefore compelled at times to head in a direction (head up) which militates somewhat against their safe delivery.

TABLE 2

Distribution of the fetuses according to side and the position of their heads

	NUMBER OF LITTERS	LEFT HORN	RIGHT HORN	DOWN	UP	UNDETERMINED
	20	90	97	94	93	0
	20	93	97	100	88	2
	20	77	95	82	89	1
	20	108	84	97	94	1
	20	93	80	95	75	3
	23	136	119	114	131	10
Total	123	597	572	582	570	17

Of the number of fetuses observed by me (1152), 570 were found with head up. Of this number, 132 were adjacent to the vagina, that is, 23 per cent—leaving 77 per cent away from the vagina. The fact that such a large proportion of fetuses with head up was found away from the vagina seems to indicate that crowding adjacent to the vagina is not the determining factor responsible for this position. Moreover, while collecting newborn rats for the study of the cervical cord, I had an opportunity to observe a number of rats during delivery, and it was noticed that a large number of fetuses were born tail first. This observation, and the fact that my data show a nearly equal number of fetuses with head up and head down, seems to indicate that this final orientation

of the fetuses has no physiological significance, but is a matter of chance.

GROWTH OF THE FETUS IN CROWN-RUMP LENGTH

Female rats of 110 to 190 days of age were mated and the time of mating recorded.[3] At suitable periods of pregnancy the rats were sent to my laboratory, where they were made unconscious mainly by means of cerebral anemia. The mother rat was then opened and the uterine horns exposed. She was then submerged in physiological salt solution, leaving the head above the level of the solution. This solution was kept at a constant temperature of 38°C. The fetuses were then shelled out of their membranes and their different physiological reactions to mechanical stimulation recorded by direct observation or by means of a motion-picture record.[4] After the fetuses had been physiologically tested, they were transferred in a spoon to standard dishes. In doing this care was taken that the fetuses floated in the solution, in order to prevent flattening or stretching of the fetus in the process of handling.

The crown-rump length of the fetus was measured with fine-pointed calipers which had a vernier attachment calibrated to $\frac{1}{50}$ mm. The measurement for each fetus was checked several times, and, in measuring, the fetuses were placed in the same position in order to eliminate possible error. The data for each horn were kept separately. Chart 3 shows the graph on the absolute growth in crown-rump length of 1157 fetuses obtained under these conditions. The ages range from eleven days after insemination to a short time after birth (twenty-two days). This material was collected from 124 litters. The dots recorded on this chart represent the average crown-rump length for entire litters, and in several instances they represent more than one litter. The crosses designate litters from defective rats, and the dot with the circle around it represents a litter with a single

[3] For the care and mating of the rats I am indebted to Miss F. L. Duhring, curator of The Wistar Institute rat colony.

[4] The results of these observations will be published later.

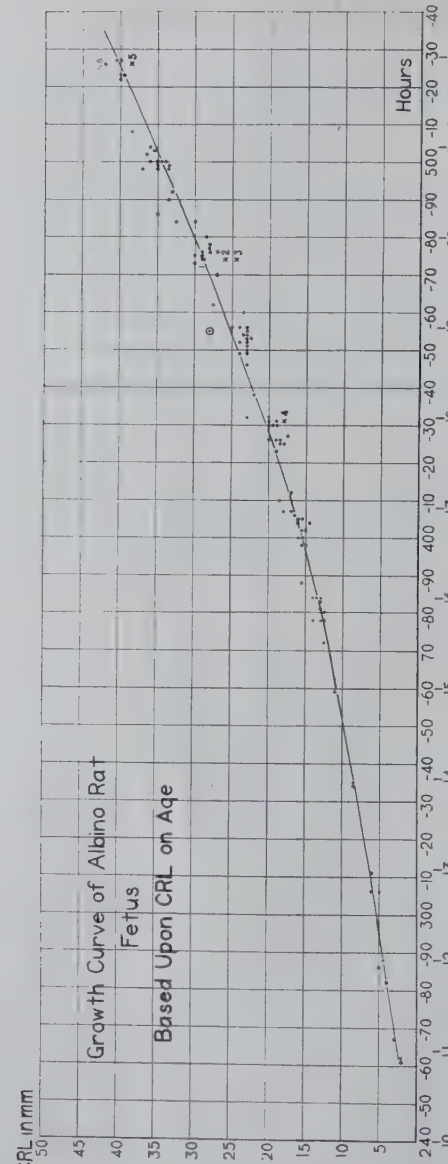

Chart 3 Shows the relation between the age and crown-rump length of the fetus. Length in millimeters is given on the ordinate and the age in hours and days on the abscissa. Each dot represents the average crown-rump length per litter. Those otherwise designated represent abnormal litters. *N.B.* stands for newborn.

fetus. According to my record, thirty-seven of these litters fall within the curve, thirty-four above, and fifty-three below. The large number of entries that fall below the curve is due to the fact that 64 per cent of them represent large litters where the number of fetuses per litter averaged above eight. This gives an indication of the effect of the litter size upon the growth of the fetuses. Of the entries that fall above and below the line, the average deviation in either case for the range of the fetal period is ± 5 per cent. However, this percentage of deviation is not uniform throughout this period,

TABLE 3

Absolute and percentage values of the daily increment in crown-rump length

AGE, DAYS	CROWN-RUMP LENGTH	ABSOLUTE GAIN	PERCENTAGE OF GAIN
11	2.0	..	..
12	4.5	2.5	125
13	6.5	2.0	44
14	8.5	2.0	30
15	11.0	2.5	29
16	13.5	2.5	23
17	16.5	3.0	22
18	20.5	4.0	24
19	25.5	5.0	24
20	30.0	4.5	16
21	35.0	5.0	16
Unborn, 22	40.5	5.5	15
Born, 22	42.6	7.6	21

as it is much larger for younger fetuses (about ± 7 per cent) and smaller for older ones (about ± 3.5 per cent). The large difference in the percentage of deviation for the younger fetuses in all probability is due to the source of error in measuring them, as they are so small that a slight error results in high values when computed on percentage basis.

This graph shows that at eleven days the average crown-rump length is 2.0 mm. This increases to 42.6 mm. at the time of birth (twenty-two days), giving a total absolute increment of 40.6 mm. The daily rate of increment of crown-rump length is given in table 3.

GROWTH OF THE FETUS IN WEIGHT

Fetuses were obtained from forty-seven litters to carry out this determination. Since the main object of my research is to determine anatomical correlations with the development of behavior, not all the fetuses could be sacrificed for this phase of the problem, which necessitates desiccation and consequent loss of the specimens for anatomical study. In most cases four fetuses were used from each litter. The selection of these fetuses was made as follows: after all the fetuses of one litter were measured the average crown-rump length was determined, and then the fetuses that came closest to this average were chosen. Thus the average crown-rump length of the selected fetuses was the same, or almost the same, as that of the entire litter. If, by reason of the small number in the litter, four fetuses could not be obtained, then the largest and smallest fetuses were selected, and thus the relationship to the average crown-rump length of the whole litter was maintained as nearly as possible. In this way the selected fetuses represent very nearly the average weight of the whole litter. The excess of water was blotted from the fetuses, and every effort was made to carry out this procedure in a uniform manner. The fetuses were then weighed in closed weighing bottles. The age of the fetuses ranged from thirteen days after insemination to a few hours after birth (twenty-two days), and the litters were so distributed in time as to show a complete record of this period of development.

The graph obtained (chart 4) represents the absolute gain in weight for fetuses from thirteen days after insemination to soon after birth (twenty-two days). The entries in this chart are indicated in the same manner as in chart 3. In addition, the entries within the squares indicate very heavy fetuses, abnormal for their respective ages. No explanation can be given for this abnormality, and since my interest in this paper is confined to the normal growth, these entries have been omitted in my computation. According to my records of the forty-two normal entries, seventeen fall in the

curve, fifteen below, and ten above. The average deviation is about $\pm$ 10 per cent. This deviation is also much larger for the younger fetuses (20 per cent) than for the older ones

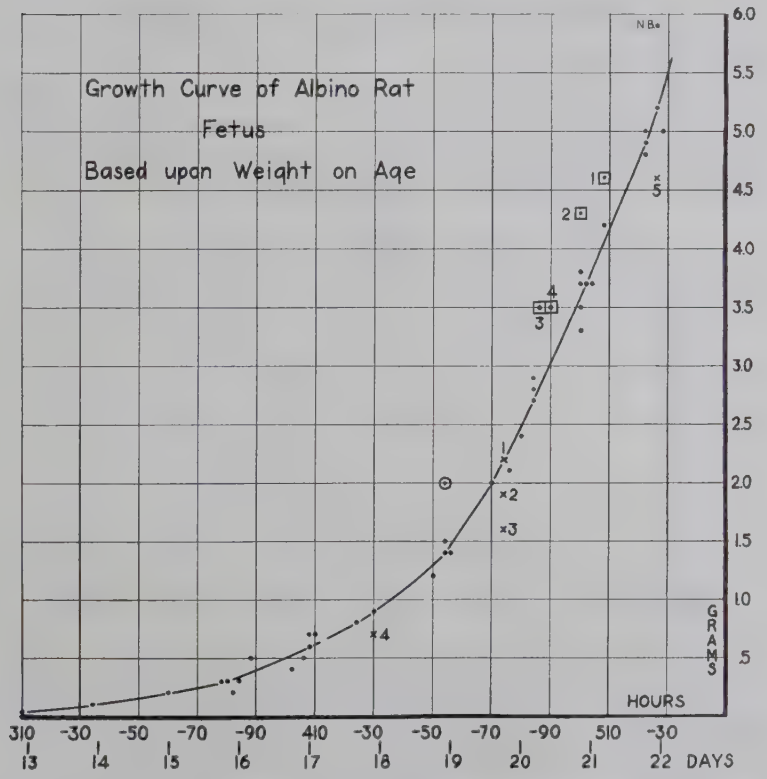

Chart 4 Shows the relation of age to the weight of the fetus. The weight of the fetus is given in grams on the ordinate and the age in hours on the abscissa. Each dot represents the average weight per litter. Those otherwise designated represent abnormal litters. *N.B.* stands for newborn.

(3.5 per cent). It should be noticed that the source of error in weighing the younger fetuses is much greater than that obtained in measuring them. In all probability this error is due to the difficulty in blotting out the excess water in a uniform manner.

This chart agrees with that of Jackson ('09) for man, Stotsenburg ('15) for the albino rat, Draper ('20) and Ibsen ('28) for the guinea-pig, and Hartman ('28) for the opossum. Thus it is seen that the curve of growth of the albino rat fetuses plotted for weight on age is, in general, similar to that of other mammals.

In chart 4 (table 4) it is seen that at thirteen days after insemination the average weight of the fetus is 0.034 gram. This increases to 5.93 grams at the time of birth (twenty-two days)—a total increment of 5.896 grams.

TABLE 4

Absolute and percentage values of the daily increment in weight

AGE, DAYS	WEIGHT	ABSOLUTE GAIN	PERCENTAGE OF GAIN
13	.034	...	..
14	.1	.066	194
15	.19	.09	90
16	.3	.11	57
17	.68	.38	126
18	.89	.21	31
19	1.43	.54	61
20	2.39	.96	67
21	3.67	1.28	53
Unborn, 22	5.09	1.42	38
Born, 22	5.93	2.26	61

If this increment or percentage of gain in weight is compared with the percentage of gain in length, it is seen that at fourteen days after insemination the percentage of increment per day in crown-rump length has decreased to 30 per cent, while that of weight is 194 per cent. Since the percentage of increment in length decreases with age, this can be interpreted as meaning that during the early part (previous to the fourteenth day) the fetuses increase in crown-rump length faster than in weight.

The curve of growth in crown-rump length based on weight is given in chart 5. The age of the fetuses in hours is recorded above the chart. In general, this curve is similar to that of Streeter (man), Latimer (cat), and Hartman (opos-

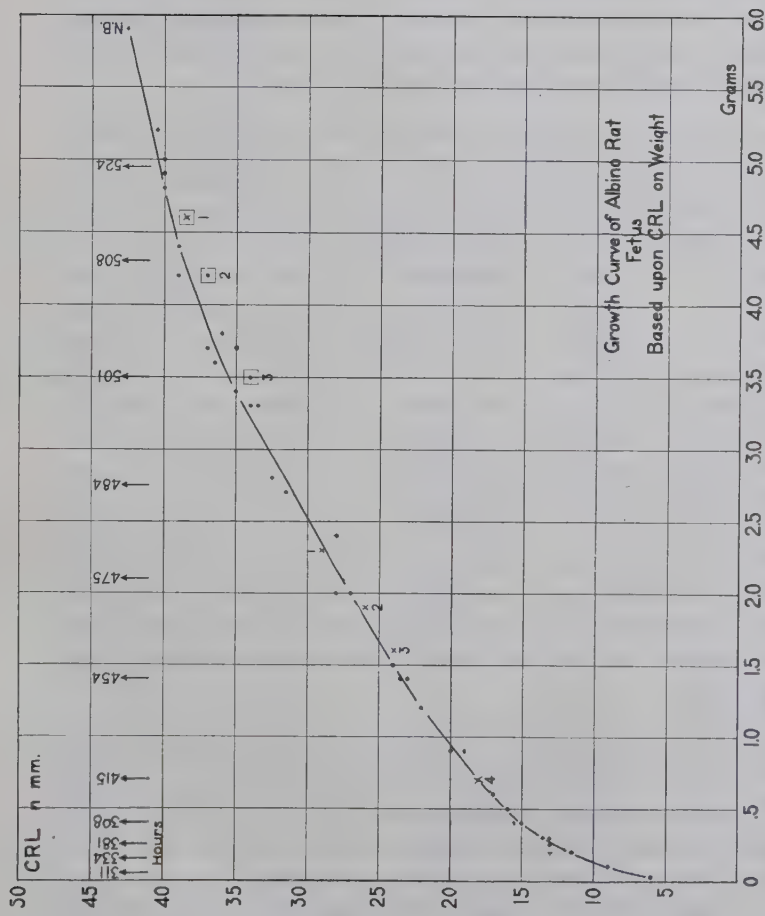

Chart 5 Shows the growth in length on weight. The length in millimeters is given on the ordinate and the weight in grams on the abscissa. The age in hours is given at the top of the chart. Each dot represents the average crown-rump length and weight per litter. Those otherwise designated represent abnormal litters. *N.B.* stands for newborn.

sum). However, if close comparison is made, one will notice that my curve rises more gradually at the beginning, and does not flatten out toward the end as do those given for man (Streeter) and the cat (Latimer). This is probably due to the fact that the material used by these authors was fixed in formalin, while the material presented in this paper was measured and weighed while fresh.

THE EFFECT OF LITTER SIZE ON THE GROWTH OF THE FETUS

The effect of the litter size on the weight of newborn albino rats has been demonstrated by King ('15); Draper ('20) and Ibsen ('28) have shown that the size of the litter affects the growth of the fetus in guinea-pigs. In these instances it was found that in large litters the average weight of the young is less than in those of small litters. In this paper the effect of litter size on the growth of the fetus is given for the entire range of fetal life. To determine this factor, the growth in crown-rump length was used as a criterion. This, I believe, gives a more nearly accurate result than the weight, because the blotting out of the excess fluid (water) in the younger fetuses can never be accomplished in a uniform manner. Also it must be kept in mind that the average weight of the fetus at thirteen days after insemination is only 0.034 gram, while the average crown-rump length at this age is 6.5 mm. Thus a slight error in the former is far more significant than in the latter.

Litters were selected that had an unequal number of fetuses in the two horns. The average crown-rump length for each horn was determined and the difference computed. The results were grouped according to age. By determining the difference between the two horns in any given rat, other factors that influence the growth of the fetuses were eliminated, leaving the litter size as the only determining factor. The result of this observation is given in table 5, which shows that from fifteen to seventeen days after insemination the effect of litter size on the growth of the fetus is almost negligible. From seventeen to nineteen days after insemination

there is a slight difference in favor of the side with the smaller number of fetuses, while from nineteen to twenty-two days a much greater difference was found. It was noticed that this difference is not equally distributed among all the fetuses, but is due to the fact that in the horns with the larger number of fetuses there is often found one or two fetuses that have a much retarded growth, while the other fetuses are

TABLE 5

Effect of litter size upon the growth of the fetuses

LITTER AGE, DAYS	NUMBER OF LITTERS	AVERAGE NUMBER OF FETUSES		AVERAGE DIFFERENCE FAVORING FETUSES IN SMALLER HORNS
		In horns with larger number	In horns with smaller number	
15–16	9	5.89	3.55	2.34
16–17	13	6.85	3.46	3.39
17–18	11	5.64	2.91	2.73
18–19	12	5.67	2.75	2.92
19–20	8	5.62	3.75	1.87
20–21	15	5.80	3.47	2.33

LITTER AGE, DAYS	AVERAGE CROWN-RUMP LENGTH FOR FETUSES		AVERAGE DIFFERENCE FAVORING FETUSES IN SMALLER HORNS	AVERAGE DIFFERENCE CORRECTED FOR A DIFFERENCE OF ONE FETUS BETWEEN THE TWO HORNS
	In horns with larger number	In horns with smaller number		
15–16	13.20	13.47	.27	.1
16–17	15.57	16.30	.73	.2
17–18	18.68	19.72	1.04	.4
18–19	23.10	24.15	1.05	.4
19–20	27.70	28.90	1.20	.7
20–21	33.90	35.80	1.80	.8

equal or nearly equal in size to those in the horns with the smaller number of fetuses.

THE EFFECT OF THE PHYSICAL CONDITION OF THE MOTHER ON THE GROWTH OF THE FETUS

The effect of the physical condition of the mother on the growth of the fetus is illustrated in charts 3 to 6. In these charts fetuses obtained from defective rats are designated by means of a cross. In charts 3 and 4, which illustrate growth on age, one notices that all but one of these entries fall much

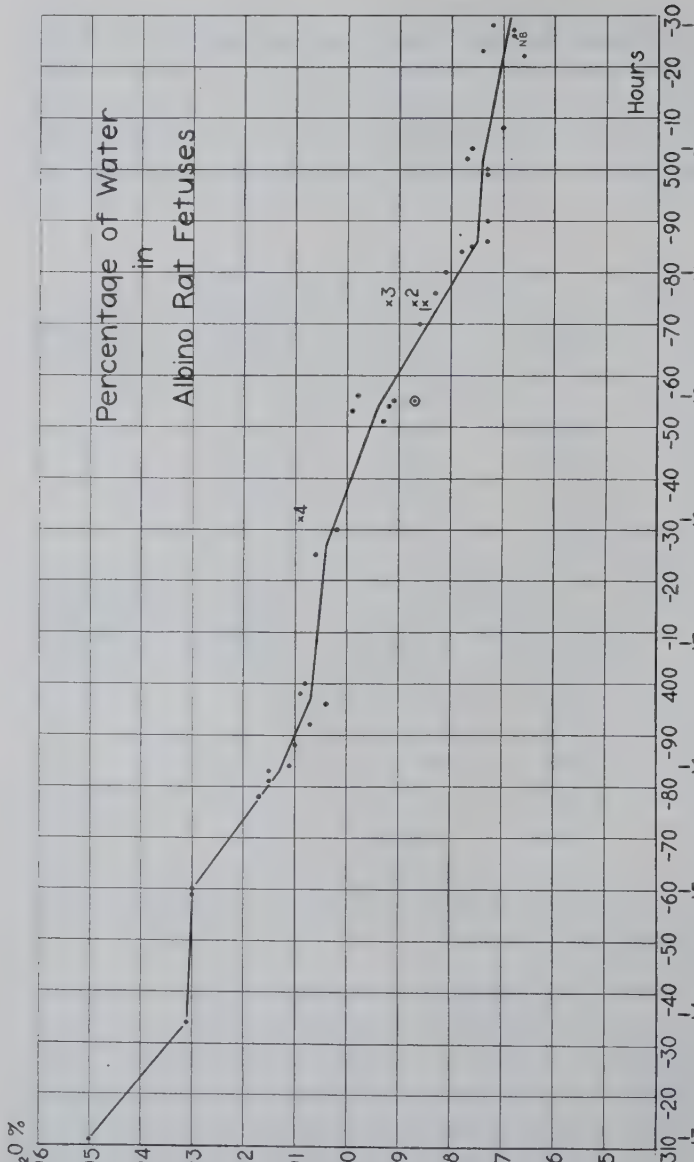

Chart 6 Shows the percentage of water in albino rat fetus. The percentage of water is given on the ordinate and the age in hours and days on the abscissa. Each dot represents the average percentage of water per litter. Those otherwise designated represent abnormal litters. *N.B.* stands for newborn.

below the mean. The different degree of deviation found for entries 1, 2, and 3 are due to the different stages of sickness of the mother. Thus in cases 1 and 2 the mother had an abscess, but in case 1 it was found that the suppurative process had stopped and that the connective tissues had completely isolated the abscess, while in case 2 the abscess was still in full suppuration. In case 3 the mother had mastoiditis. Cases 4 and 5 represent mothers with abscess in full suppuration. It should be noticed that in chart 6 these entries fall above the mean. Furthermore, cases 1, 2, 3 in this chart show the same relationship above the mean as they show in charts 3 and 4 below the mean. In other words, there is a correlation between the degree of arrested growth and the degree of excess of the percentage of water. And since, as explained below, the percentage of water indicates the degree of differentiation, this indicates that the fetuses obtained from defective mothers were not only arrested in growth, but also arrested in differentiation.

PERCENTAGE OF WATER

The fetuses used to determine the growth in weight were also used for this study. After the fetuses were weighed in the fresh condition, they were placed in a drying oven at a temperature of 97° to 98°C., and were then weighed at weekly intervals, if the weather so permitted, until there was no more reduction in weight. This took place usually in three weeks' time.[5]

In the albino rat the percentage of water decreases in the central nervous system from birth to old age (Donaldson, '10). A similar decrease takes place in the cartilaginous skeleton (Donaldson and Conrow, '19). Also the percentage of water decreases in the whole body with the increase of age (Hatai, '17). The result of my observation in the albino rat fetus shows that there is a reduction in the percentage of

[5] Excessive humidity in the air causes the material to absorb water, thus increasing the weight. For this reason weighing was done only when the humidity was about normal.

water in correlation with the increase of age of the fetus (thirteen days after insemination to the time of birth (twenty-two days); table 6, chart 6). This indicates that the percentage of water in the fetus is a function of age, as demonstrated by Doctor Donaldson ('10) for the nervous system

TABLE 6

Percentage of water in albino rat fetuses

AGE, HOURS	FRESH WEIGHT	TOTAL AMOUNT H_2O	PERCENTAGE OF H_2O
311	.034	.032	95.0
334	.10	.09	93.1
359	.19	.17	93.0
378	.28	.25	91.7
381	.30	.27	91.5
383	.24	.22	91.5
384	.30	.28	91.1
388	.47	.42	91.0
402	.45	.40	90.7
406	.48	.44	90.4
408	.68	.62	90.9
410	.74	.68	90.8
425	.77	.70	90.6
430	.89	.80	90.2
451	1.24	1.16	89.3
453	1.46	1.32	89.9
454	1.53	1.36	89.2
455	1.44	1.28	89.1
456	1.43	1.28	89.8
470	1.97	1.74	88.6
476	2.07	1.83	88.3
480	2.39	2.11	88.1
484	2.75	2.42	87.8
485	2.86	2.51	87.6
486	3.52	3.07	87.3
490	3.50	3.06	87.3
499	4.00	3.50	87.4
500	3.48	3.04	87.3
502	3.74	3.28	87.7
504	3.67	3.22	87.6
508	4.39	3.82	87.0
522	5.02	4.35	86.6
523	4.84	4.23	87.4
527	5.09	4.43	87.0
526	5.93	5.15	86.8 (Newborn)

of the albino rat from birth to maturity. However, it should be noticed that the graph representing this decrease—tabulated percentage of water on age—for the albino rat fetus does not follow the same general pattern as that given for the central nervous system, cartilaginous skeleton, and the whole body of the albino rat from birth to old age. In the latter cases the reduction of the percentage of water takes place almost wholly within the first third of the life span. Thereafter the reduction is almost negligible. In the former case the reduction in the percentage of water is distributed wthin the entire range of the fetal period. Also, from birth to maturity the decrease in the percentage of water in the brain is continuous and progressive except during the first eight days after birth (Donaldson and Hatai, '31), while during the fetal period there is a strong indication of periodicity in the decrease of the percentage of water.

In the albino rat the period of rapid decrease of the percentage of water in the nervous system coincides with the period of myelination (Donaldson, '16, '16 a). A rapid reduction in the percentage of water has also been recorded for the several anatomical systems which compose the body (Lowrey, '13). The process of myelination can be considered as a highly specialized type of cell differentiation characterized, as pointed out by Donaldson ('16), by the ability of the nerve cell to throw out around the axons the lipoids (or nonprotein substance) which remains bound within the cells in the other tissues. The period from birth to the one hundredth day in the albino rat can also be considered as a period of maturation (differentiation) of all the tissues. From this we can conclude that differentiation is characterized by a reduction in the percentage of water. Therefore, the periods of reduction in the percentage of water illustrated in chart 6 may indicate periods of cell differentiation, and it is possible that the periods in which the percentage of water remains stationary indicate periods of cell proliferation. Thus from thirteen to fourteen days after insemination there would be, according to this hypothesis, a period when cell differentia-

tion predominates, the cells during this period increasing in size, adding solids to their protoplasm, and thus bringing about a relative reduction in the percentage of water (chart 6). Also this would be followed by a period when cell proliferation predominates, the percentage of water remaining stationary—fourteen to fifteen days after insemination. From fifteen to sixteen and one-half days there would be another period of cell differentiation, followed by another period of cell proliferation from sixteen and one-half to eighteen days. This would be followed by a long period of cell differentiation which lasts from eighteen to twenty days, and then a short period of cell proliferation, followed again by a period of cell differentiation up to the time of birth (twenty-two days).

The significance of this periodicity in the reduction of the percentage of water will be further examined in connection with my studies on the correlation of structure with the development of motility in the albino rat.

SUMMARY

The prenatal growth of the albino rat has been studied in a well-graded series that range from eleven days after insemination to the time of birth (twenty-two days). The material was standardized in accordance with the factors enumerated by Dr. Helen Dean King ('15), excepting the litter size, which is beyond experimental control.

The effect of litter size upon the growth of the fetus is in direct proportion to the age of the fetus, being negligible during the early period of growth up to the seventeenth day after insemination, slightly affecting the growth of the fetus from the seventeenth to the nineteenth day, and showing a more marked effect toward the end of the gestation period. It manifests itself more markedly upon a few members of the litter.

When the material is standardized as described above, the results obtained are very uniform. The slight variation noticed in this series could be attributed in a large measure to the effect of litter size.

The distribution according to side and position of the head is almost equal, suggesting that the distribution and orientation of the fetus are a matter of chance and have no physiological significance.

The percentage of increment in length decreases uniformly with age, while the decrease in the percentage of increment in weight is not uniform.

The percentage of water decreases in a periodical fashion, being in a large measure a function of age and the degree of differentiation of the fetus.

The necessity of using standardized material for this type of research, as well as for anatomical and behavioral studies, is emphasized.

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A VENTRAL ACCESSORY TAIL IN TRITURUS VIRIDESCENS AND ITS DUPLICATION EXPERIMENTALLY

ALDEN B. DAWSON

Zoölogical Laboratories, Harvard University

THREE TEXT FIGURES AND TWO PLATES (SEVEN FIGURES)

The description of an anomalous development occurring in nature usually proves to be more or less unsatisfactory, since only the end results are known to the investigator, and, in the absence of any definite knowledge regarding the causative factors involved, it is always necessary to make certain assumptions if any explanation of the atypical structure is to be offered. In some cases the results of experimental studies may clearly indicate the nature of the factors producing the abnormality and, conversely, the character of the anomaly frequently furnishes additional information on the potentialities of development.

The ventral accessory tail, the subject of this report, is of interest chiefly on account of the relationship of the spinal cord to the axial skeleton. An isolated segment of typically developed cord, with dorsal ganglia present, occupies a normal position in relation to the vertebrae of the accessory tail, but is separated from the normal spinal cord by the continuation of the main axial skeleton. It seems probable that the accessory tail is a result of regenerative processes following an injury or successive injuries.

DESCRIPTION

The specimen, a male, was found among a shipment of animals sent from the vicinity of Northampton, Massachusetts, through the kindness of Miss Madeléine Grant. The animal,

with the exception of the ventral accessory tail, was normal in all respects. The extra tail was small and lay almost entirely within the ventral fin of the main tail. Its presence, however, caused a considerable regional expansion of the ventral margin of the fin. The thin margin of this expansion which can probably be regarded as the fin of the extra tail was bent sharply to the right and then directed dorsally (fig. 11). This atypical position was probably caused by contact with the substratum on which the animal lived.

When examined in brilliant, transmitted light, the axial skeleton of the accessory tail was found to consist of six vertebrae (fig. 8). Three of these lay immediately ventral to the normal axial skeleton. They were not opposite the normal vertebrae, but alternated with them. The remaining three vertebrae diverged rapidly from the main axial skeleton and were directed obliquely toward the ventral margin of the normal tail. Anterior to the region of doubled vertebrae the caudal blood vessels bifurcated, one set passing to the left of the accessory axial skeleton to supply the main tail, the other passing ventrally to accompany the vertebrae of the extra tail.

The portion of the tail shown in figure 8 was sectioned transversely. Both anterior and caudal to the region of the accessory tail such axial structures as spinal cord, vertebral column, caudal vessels, and musculature were normal. Owing to the extreme irregularity of the caudal vertebrae of the Salamandridae and the practical absence of any definite centra, little exact information on the form and relationship of the bony parts of the vertebrae could be obtained from a study of transverse sections without resorting to reconstructions. A more definite idea of the extent of reduplication, however, is obtained from a study of the intervertebral cartilaginous zones between the ends of the centra (fig. 9). Since the vertebrae in the region of reduplication do not lie opposite each other, but have an alternating arrangement, the corresponding intervertebral cartilages are not in the same section. Nevertheless a definite doubling is apparent and it

is clear that the ventral vertebrae are fused more or less completely with the dorsal segments of the main tail. Accordingly, the neural arch is absent from these ventral vertebrae of the accessory tail.

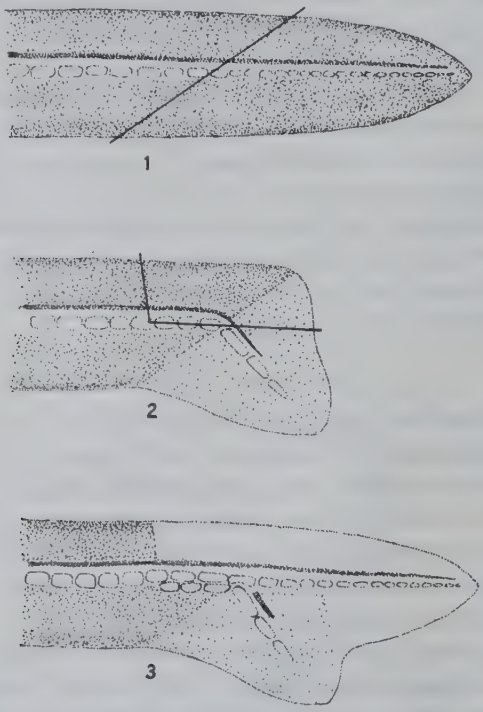
The three other vertebrae which diverge from the main axis and lie in the ventral fin possess a definite neural arch and appear to be normal in all respects (figs. 10 and 11). The first of the three divergent vertebrae is accompanied by an isolated segment of typical spinal cord, and paired dorsal ganglia are present in this region. The accessory spinal cord ends abruptly and no central nervous tissue is found beyond this first vertebra. A typically developed axial musculature, however, envelops all three vertebrae. The epidermis of the integument over the tip of the last vertebra is thickened, deeply pigmented, and cornified.

INTERPRETATION

It is difficult to interpret the ventral tail as a result of simple regeneration following a single injury. The presence of the isolated portion of spinal cord which accompanies its axial skeleton would almost preclude such an explanation. It seems necessary to assume a second injury occurring after the regenerative process initiated by the primary injury had progressed some considerable distance.

If, in the first injury, the tail of the newt had been cut off obliquely as suggested in figure 1, the new tail would at first stand at right angles to the oblique surface. If no subsequent injury occurred, the new tail as it became larger would gradually swing around into line with the old part. Accordingly, it is necessary to assume that the tail suffered a second injury before this realignment of the new part with the old had taken place. The second injury would have to involve the dorsal margin of the tail and include portions of both the old and new parts. It would also have to be deep enough to remove a segment of the spinal cord and the dorsal portions of several vertebrae of the old part (fig. 2). The regeneration following the second injury could conceivably result in

the restoration of the main axis of the animal. These assumptions would explain the region of doubled vertebrae, the small accessory tail, and the isolated segment of typical spinal cord present in this extra tail.



Three diagrams to illustrate the assumptions made in the text to explain the development of the accessory ventral tail.

Fig. 1 The probable direction and extent of the first injury.

Fig. 2 The regenerated tail which would develop at right angles to the oblique surface. The heavy line illustrates the probable extent of the second injury.

Fig. 3 The subsequent regeneration which might result in the restoration of the main axis and the isolation of a portion of spinal cord.

EXPERIMENTAL VERIFICATION

After the preceding section was written, it was decided to test the hypothesis experimentally, and a number of animals were subjected to the operative procedures just outlined. A

variety of results were obtained, due chiefly to the difficulty in carrying the second cut to the proper depth. If a complete portion of the spinal cord were not removed, healing of the wound without regeneration followed and the new ventrally directed tail eventually became realigned with the main axis of the body. If the cut were carried too deep into the centra of the vertebrae, the newly regenerated tail was sloughed off and a simple terminal regeneration resulted.

However, when the cut was made at the proper depth the regenerating ventral tail persisted and subsequent regenerative activity resulted in the reestablishment of the main axis by the production of a second, more dorsal tail. Three of the most successful experiments are illustrated in plate 1 (figs. 4, 5, and 7).

Histological examination of these tails showed that an isolated portion of spinal cord was associated with the ventral tail. In these experimental animals the central nervous tissue, which normally extends to the end of the skeletal axis, was lacking at the extreme tip of the ventral tail, but did not end so abruptly as in the animal found in nature. In all other respects the conditions in the several tails were found to be practically similar.

After a successful second operation, there did not appear to be any further proliferation of the axial skeleton of the ventral tail. The vertebral primordia, laid down in cartilage, continued to grow and differentiate, eventually becoming ossified, but there was no additional segmentation of the cartilaginous axis. Moreover, the caudal extension of the isolated segment of spinal cord also appeared to be retarded. Growth in length did not take place as rapidly as in the elements of the axial skeleton, resulting in the absence of spinal cord at the extreme tip of the accessory tail.

The retardation of growth in length observed in the ventral tail was not apparent in the dorsal tail which was developing in line with the main axis of the animal. The amount of caudal extension of the dorsal tail is apparently dependent on the level at which the tail was originally cut off and

tended to come to a standstill when the original length of the animal was restored. This is illustrated in figures 5, 6, and 7. In figure 5 the ventral tail had been growing for 118 days and the dorsal tail only ninety-six days. Furthermore, the dorsal tail began its regenerative activity at a point some three vertebrae more cranial. The same differences in times and levels of regenerative activity hold for the specimen shown in figures 6 and 7. In figure 6 the relative amount of growth of the two tails is shown seventy-eight days after the first amputation, or fifty-six days after the second operation. In figure 7 the same animal is shown thirty-six days later. The ventral tail is practically unchanged, while the dorsal tail has considerably increased in length.

The spinal cord of the ventral tail was isolated at the time of the second operation, but the continuity of its skeletal axis was maintained and the blood supply (ventral caudal vessels) was not disturbed. In the dorsal or second tail, the axial skeleton, nerve cord, and blood vessels developed as the tail grew out and all three structures were present during development. Accordingly, the interruption of the spinal cord of the ventral tail constitutes the chief anatomical difference between the two tails. Whether this defect alone was responsible for the retardation of growth or whether the atypical ventral position and the consequent limiting effect of the ventral border of the caudal fin may be additional retarding factors it is difficult to determine.

The relative importance of nerve cord and skeletal axis in normal regeneration is still undecided. Aron ('29), on the basis of his experiments on anuran larvae, recognizes two regions in the spinal cord, a postcervical and a precaudal, which exercise a definite influence on axial growth. Transections of the cord which isolate the tail from the precaudal growth center result in retardation of growth and atypical development of this portion of the animal. Locatelli ('29) also found, in Triton in the absence of spinal cord, that regeneration of the tail was inhibited. She did not regard this as a direct result of the absence of central nervous tissue,

but concluded that the inhibition of regeneration was due rather to lack of spinal ganglia which normally would develop from the spinal cord. The present experiments do not permit of an analysis of the relative importance of spinal ganglia and spinal cord, since ganglia, in association with a segment of spinal cord, were regularly present in all accessory tails. Accordingly, so far as could be determined, the interruption of the spinal cord and the atypical position of the ventral tail were the only factors which limited its progressive development. The dorsal tail developed at a rate comparable to that of the regeneration of tails following simple section.

As previously pointed out, the chief features of interest in the abnormal tail found in nature were that the accessory tail was ventral in position and possessed an isolated segment of spinal cord. Dorsal accessory tails of a similar nature were produced by Barfurth ('99) in tadpoles; and Gräper ('09) described a three-tailed lizard with a total of seven axial skeletons and supernumerary spinal cords which he interpreted as due to successive dorsal and ventral injuries involving both the vertebral column and spinal cord, followed by regeneration from the exposed ends on both the cranial and caudal margins of the wounds. Accordingly, duplicate tails may arise in several ways, but the interpretation of the manner in which the accessory ventral tail in Triturus was developed fits the anatomical findings. Moreover, similar tails were produced experimentally.

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PLATE 1

EXPLANATION OF FIGURES

4 Tail cut obliquely, May 1st, recut on May 19th, and photographed on June 2nd. Following the removal of the block of tissue in the second operation the regenerating tail swung dorsally 90°, but as regeneration from the main axis proceeded it was gradually forced ventrally. Its presence, however, produced a slight distortion of the tip of the dorsal tail.

5 Tail, cut obliquely on July 7th, recut on July 29th, and photographed on November 2nd. The relationship established between the two tails is very similar to that of the specimen found in nature (fig. 8).

6 and 7 Tail, cut obliquely on July 7th, recut on July 29th, photographed first on September 23rd (fig. 6) and later on October 29th (fig. 7). Compare with figures 5 and 8.

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PLATE 2

EXPLANATION OF FIGURES

8 A portion of the abnormal tail, viewed from the left side, showing the relationship and extent of the accessory tail which lies almost entirely within the main ventral fin. The axial skeleton of the accessory structure consists of six vertebrae, three intimately related to, but alternating with, corresponding segments of the main axial skeleton and three which diverge ventrally to lie in the loose connective tissue of the fin. The main caudal blood vessels pass to the left of the accessory tail after giving off branches to supply this structure. (Living specimen, photographed with transmitted light, × 4.)

9 A section transverse to the axis of the main tail, passing through the second accessory caudal vertebra, showing the spinal cord and the double axial skeleton. Two sets of caudal blood vessels are shown, one set lying to the left of vertebral column (right side in the photograph) and one ventral to it. The body musculature appears normal at this level.

10 A more caudal section passing obliquely through the first of the divergent accessory, caudal vertebrae. The main axial elements lying in a more dorsal position are not included in the photograph. The isolated portion of spinal cord associated with the extra tail has a well-defined central canal. On the left side (right in the photograph) a portion of a dorsal ganglion is shown. A well-developed axial musculature accompanies the extra vertebra. The ventral fin is slightly deformed at this level and curves to the left.

11 A still more caudal section passing almost longitudinally through the two terminal vertebrae of the extra tail. A well-developed axial musculature is present. The thin ventral edge of the tail is bent sharply to the right and turned directly dorsally. The reflected portion is probably the fin of the extra tail. The position it assumes is possibly due to the fact that it projected beyond the ventral margin of the normal fin and was deformed by contact with the substratum on which the animal moved. The epithelium over the end of the last accessory vertebra is thickened and the more superficial layers are cornified.

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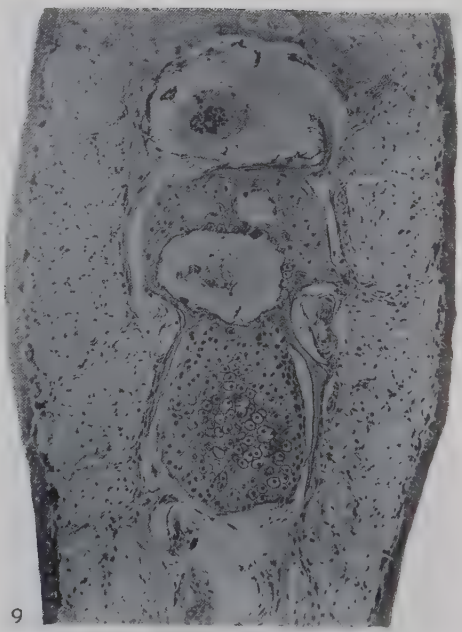

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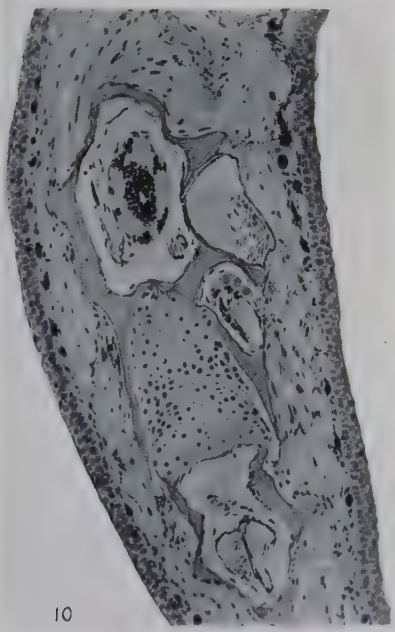

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UNUSUAL STRUCTURES IN THE ERYTHROCYTE[1]

II. A PRECISE NUCLEAR IMPREGNATION METHOD

JAMES F. RINEHART

San Francisco, California

TEN FIGURES

In this communication there will be described and discussed an unusual 'architecture' revealed in the erythrocyte by a special technical method. In the course of an experimental study(1) for the precipitation and silver impregnation of hemoglobin, a procedure was evolved which revealed a surprising series of structures in the erythrocyte. The method has not only a unique action on the erythrocyte, but also stains precisely the nuclei of the white blood cells. The method involves a preliminary mordanting of a dried and methyl-alcohol-fixed blood film in a solution of reduced phosphomolybdic acid and phosphotungstic acid and subsequent impregnation with silver ammonium carbonate. The detail of this procedure, which will be referred to as method A, is given in the following section.

TECHNIQUE

The technical method employed involves the use of reagents and equipment similar to those used in the method recently described by the author(1) for precipitation and impregnation of hemoglobin. For the convenience of the reader the detail is given below.

The procedure here employed is exacting and requires attention to details. Several more or less unstable reagents are employed which may render results somewhat variable. Minor difficulties common to all methods involving silver impregnation are encountered. It is not

[1] From the Thorndike Memorial Laboratory and the Second and Fourth Medical Services (Harvard) of the Boston City Hospital, and the Cancer Commission of Harvard University.

improbable that minor variations in technique may improve results, but the method set forth has, in the author's hands, given precise pictures.

Glassware

Scrupulous attention to the chemical cleanliness of the glassware in this, as in other silver technique, is rewarded by improved results.

Blood films

Cover-slip blood films are made in the usual manner on chemically clean cover-glasses. An evenly spread, thin but untraumatized film is essential. These are air-dried. Best results are obtained if the film is stained within twenty-four hours after drying.

Chemicals required

The essential reagents required are the following: 1) Molybdic-acid reagents of Folin(2).[2] 2) Sodium sulphantimonate. 3) Phosphotungstic acid (C.P.). 4) Silver nitrate (reagent). 5) Anhydrous sodium carbonate (C.P.). 6) Ammonium hydroxide (C.P.). 7) Formalin (reagent). 8) Glacial acetic acid.

Reagents employed

The reagents actually employed in the procedure are as follows: 1) Partly reduced molybdic-acid reagent (solution B). 2) 5 per cent aqueous solution of phosphotungstic acid. 3) Silver ammonium carbonate. 4) 10 per cent aqueous solution of formalin. 5) Glacial acetic acid.

[2] "For the preparation of the purified reagent it is advantageous to keep on hand a brominated 30 per cent solution of sodium molybdate. By means of a large funnel and fine glass rod transfer 600 gms. of sodium molybdate to a 2 liter volumetric flask. Add much water and shake until solution is complete except for a slight turbidity. Dilute to volume, mix, and transfer this stock solution to a large flask or bottle. Add about 0.5 cc. of liquid bromine, shake, and set aside. Transfer 500 cc. of the clear supernatant solution to a Florence flask, capacity 1000 to 1500 cc. Add with stirring 225 cc. of 85 per cent phosphoric acid. Some bromine is set free and imparts a yellow color to the solution. Next add 150 cc. of cool sulphuric acid (25 volumes per cent). Remove the bromine by means of an air current either immediately while the solution is still warm or, better still, the next day. Then add 75 cc. of 99 per cent acetic acid. Mix and dilute to 1 liter. If protected from organic matter, this reagent will remain colorless for months." Only the best C.P. grades of chemicals should be used in the preparation of this reagent.

Preparation of the reagents

1. Reduced molybdic-acid reagent—solution B. The reduced molybdic-acid reagent is prepared by adding 100 cc. of a freshly prepared 3 per cent solution of sodium sulphantimonate to 100 cc. of the Folin molybdic-acid solution. This is best done by allowing the sodium sulphantimonate solution to drop from a funnel filter into a dish containing the Folin reagent, which should be stirred constantly until all the antimony salt has passed through the filter. The molybdic-acid solution immediately turns blue on reduction and a heavy, blue-black precipitate forms. The precipitate is removed from the solution by gravity filtration through a filter-paper that will remove fine precipitates. The deep blue filtrate forms a stock solution, which we will refer to as solution B. This solution should be entirely free of precipitate. Results will depend to a considerable extent upon satisfactory preparation of this solution. Perhaps, due to variability in the reducing capacity of the sodium sulphantimonate, the preparation of solution B is not absolutely uniform. Variability in this solution will, to some extent, be reflected in results. This solution should be kept in a glass-stoppered bottle. The author has been able to trace most difficulties encountered to this solution. A poorly prepared or deteriorated solution B should be discarded.

2. Prepare a 5 per cent aqueous solution of phosphotungstic acid.

3. Silver ammonium carbonate.

The preparation of the silver ammonium carbonate must be done carefully. The quickest and the most accurate method appears to be the one in which balanced molecular proportions are used as advised by Kubie and Davidson(3) and applied by Foot(4) in the impregnation of neuroglia. The author uses a concentration stronger than that employed by Foot.

The dilutions used in this study are as follows:

1) 20.4 per cent $AgNO_3$ (1.2 molar)
2) 8.1 per cent NH_4OH (1.2 molar) (Ammonium hydroxide containing
3) 6.2 per cent Na_2CO_3 (.6 molar) 28 to 29 per cent NH_3)

We see by the formula $(Ag(NH_3)_2)_2CO_3$, for silver diammino carbonate, that one molecular proportion of silver nitrate requires two proportions of the ammonium hydroxide, and this compound ion $(Ag(NH_3)_2)^+$ in turn requires one-half the molecular value of sodium carbonate. Exactly 10 cc. of the 20.4 per cent $AgNO_3$ is transferred by pipette into a 50-cc. glass-stoppered graduate. To this is added about 19 cc. of the 8.1 per cent NH_4OH, somewhat less than the amount required to clear the solution. A heavy brown precipitate is formed. Then the 8.1 per cent ammonium hydroxide is added a drop at a time from a dropping bottle, with intermittent

shaking until the precipitate just disappears. Care should be taken that this end point is not passed. To this solution exactly 10 cc. of the 6.2 per cent Na_2CO_3 is added. There is no further precipitation. This solution of silver diammino carbonate is not stable and should be made up fresh each day that it is used and unused portions discarded. To be sure there is no precipitate, the author usually filters the solution through a paper that will remove fine precipitates into the flat (McJunkin) staining dish just before use.

4. Prepare a 10 per cent aqueous solution of reagent neutral formalin.

Technique of staining; method A

Five cubic centimeters of solution B is mixed with 4 cc. of 5 per cent phosphotungstic-acid solution in a flat McJunkin staining dish, to which is added eight to ten drops of glacial acetic acid. The dish is placed in an incubator at 40° to 45°C. for ten minutes or more.

The dried cover-slip blood film is placed on a cork and methyl alcohol poured upon the film surface. After about five minutes this is poured off, a fresh solution poured on and quickly drained off. The film is placed in the incubator, and when it has just become dry, it is submerged in the prepared solution with a clean glass rod. The film is left in this solution from thirty to forty minutes.

After this interval the dish is removed from the incubator. The cover-glass films are picked up individually with a pair of fine-pointed forceps in the right hand and quickly but thoroughly washed by passing, with three waving motions, through a beaker containing about 400 cc. of distilled water. The film is then transferred to a pair of forceps held in the left hand and similarly washed through a second beaker of distilled water and then passed directly into a staining dish containing about 10 cc. of the freshly prepared silver ammonium carbonate. This dish is then placed in the incubator at 40° to 45°C. and allowed to remain from ten to twelve minutes. Transfer from the silver bath through two beakers of distilled water is accomplished in the manner described for previous transfer. From the second distilled-water wash, the blood film is passed into a staining dish containing about 20 cc. of a 10 per cent solution of reagent neutral formalin. This reducing bath probably is improved by adding a small amount of sodium carbonate to the formalin. The author uses two drops of the 6.2 per cent solution of sodium carbonate employed in the preparation of the balanced silver ammonium carbonate.

The film when passed into the formalin assumes a deep brown tone. Reduction is almost instantaneous. It is well to shake the dish containing the formalin as the preparations aare passed into

the solution. The blood film is removed from the formalin, rinsed in distilled water, blotted, dried, and mounted in neutral balsam.

The combined method

The combined method used in this study is accomplished by introducing one more step in the procedure. After the sections have been acted upon for the desired period in the phosphotungstic and reduced phosphomolybdic-acid reagent (solution B), they are passed, after washing in the manner described, into the solution for precipitation of homoglobin. This is simply prepared by mixing 5 cc. of solution B and 5 cc. of 5 per cent potassium dichromate. (These solutions should be mixed and placed in the oven about ten minutes before use.)

When the sections have been in this solution for ten minutes, they are washed and passed into the silver bath for ten minutes, washed again and reduced in 10 per cent formalin, washed, dried, and mounted in balsam.

THE STRUCTURE REVEALED (Method A)

When the procedure is satisfactorily executed, clearly outlined round or oval bodies are seen in the erythrocytes which present such distinctness and regularity that they suggest an 'organ' or apparatus previously undescribed and peculiar to the cell.

The oval or round bodies appear in various positions in the erythrocyte and are seen to be composed of fine metachromatic granules of greenish or reddish tone. The appearance of the architecture in question is illustrated in the accompanying photomicrographs.

Figure 1 illustrates the appearance in normal blood. The regular distribution and precise outline of the structures are readily seen. The ground substance in which the bodies lie is usually of a clear yellow or yellow-green tone, but may be slightly granular in appearance.

Young cells or reticulocytes show a dark brownish opaque ground substance, suggesting a difference in physical constitution of the ground substance (fig. 2). Figure 3 shows the appearance of the erythrocytes from a case of pernicious anemia in relapse. Nucleated human erythrocytes may show from one to several bodies.

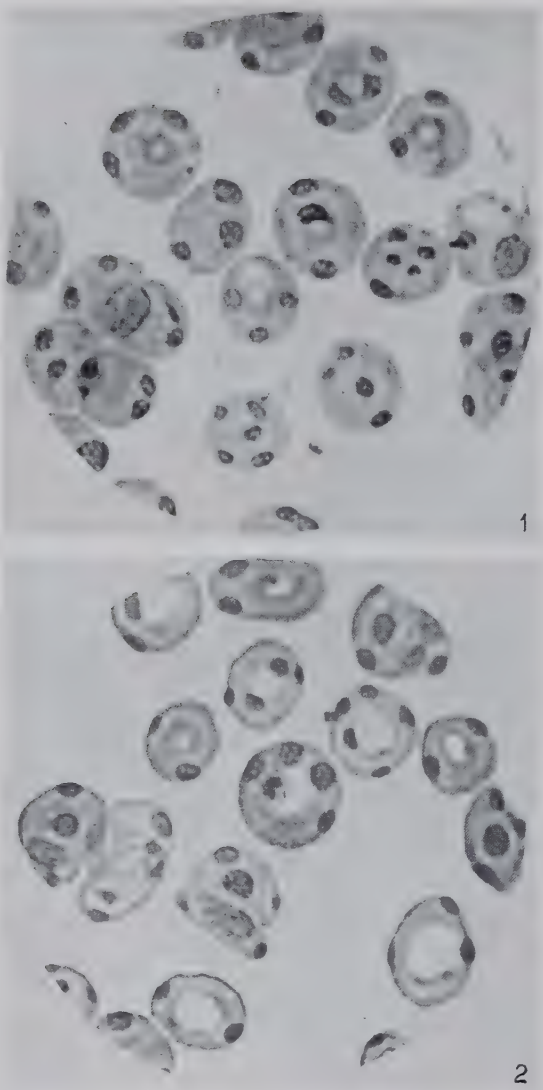


Fig. 1 Normal erythrocytes showing precise arrangement of bodies composed of metachromatic granules in smoothly outlined cells. There is a very fine granularity of the greenish yellow background. Method A. × 1750.

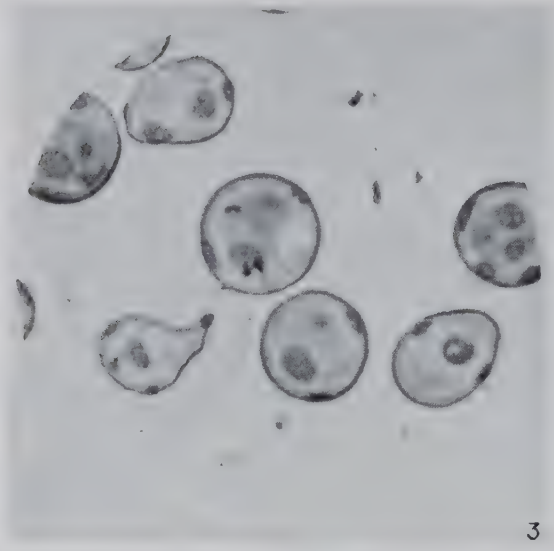
Fig. 2 Erythrocytes from a case of secondary anemia. The larger cell in the center contains the dull brown granular ground substance characteristic of young cells. Method A. × 1750.

THE NATURE OF THE 'STRUCTURES'

The surprising and unexpected appearance of these bodies in a cell generally considered of a rather simple constitution, together with certain variations in the appearance of the 'structures' in different preparations and different portions of the same preparation, led the author to search for some explanation of the structures on an artificial basis. Among the observations suggesting that the bodies were not preformed structures was the occasional finding of granular material, similar to that forming the structures, lying outside the cells; and sometimes it appeared that the granule-composed bodies were extruded beyond the general contour of the cell (fig. 7). Furthermore, in cells that had undergone some crenation, it appeared that the outline of the 'architecture' formed in the cells was determined by this, i.e., the granular material appeared in the ridges and the nodal points of the crenated surface (fig. 4). An explanation of the nature of the structures, it is believed, was afforded when the method described in this paper was used in combination with the method recently described by the author for the precipitation and impregnation of hemoglobin(1). Dried and methyl-alcohol-fixed blood films were carried into the solution of reduced phosphomolybdic acid and phosphotungstic acid and then passed into the solution for precipitation of hemoglobin and subsequently impregnated with silver ammonium carbonate. The reagent used for the precipitation of hemoglobin consists of a mixture of reduced phosphomolybdic acid and potassium dichromate. This procedure will be referred to as the 'combined' method, the detail of which is given under the section on methods.

When the blood film is passed into the dichromate and reduced phosphomolybdic-acid mixture, the hemoglobin is precipitated in a granular form in the position occupied by it at the moment. Subsequent application of the silver ammonium carbonate impregnates this precipitated substance.

When this combined technique is applied it appears that the stages in the formation of the peculiar 'architecture' of



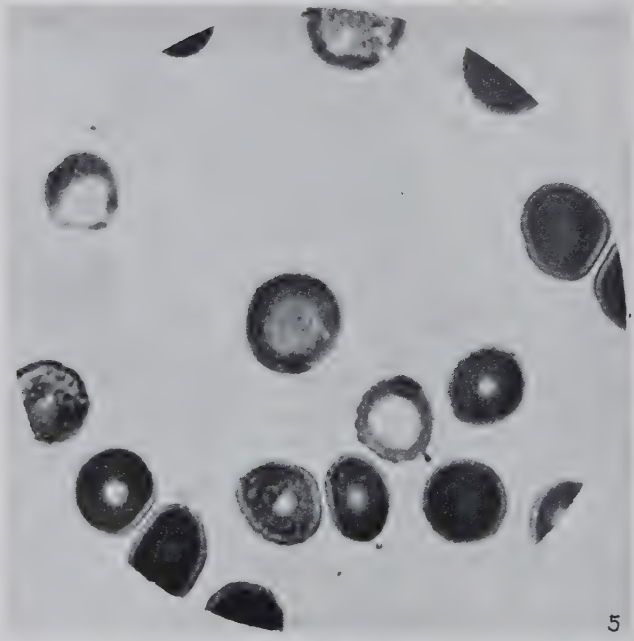
Figures 3 and 4

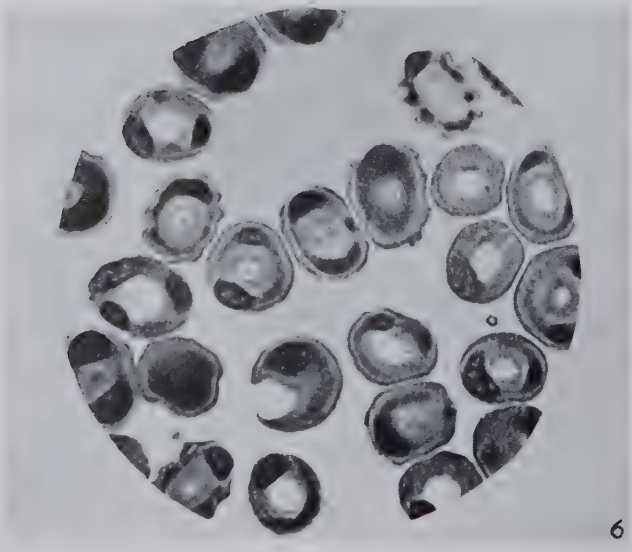
the red blood cells can be followed. Many of the cells show the completely formed oval or round bodies in an otherwise clear cytoplasm. In other cells, however, a fine granular precipitate extends between imperfectly outlined bodies or forms a peripheral rim without distinct segregation into bodies. Still other appearances encountered are a shift of the granular material (hemoglobin) to one side of the erythrocyte or to a position in the center of the cell. In some instances the fine granules are seen uniformly distributed through the cell. In these cells there has, apparently, been no dissociation of the hemoglobin. If the preliminary treatment in the phosphotungstic acid and reduced phosphomolybdic acid is shortened in the combined method, a larger percentage of the cells show the undissociated uniform distribution of granules and a few show the onset of the formation of the oval structures.

Figure 5 shows the erythrocytes in case of mild 'secondary' anemia treated by the combined method with a short period of only ten minutes in the bath of phosphotungstic and reduced phosphomolybdic acid and subsequent treatment in the hemoglobin-precipitating agent. It will be seen that most of the cells are diffusely filled with finely granular dark substance; a few show some concentration of the granular material toward the periphery with an early stage of the structure formation. Figure 6 shows the cells in a blood film from the same patient treated similarly, but for a longer period, in the phosphotungstic and reduced phosphomolybdic-acid bath. The structures are more distinctly formed, some more defi-

Fig. 3 Showing the appearance of the 'structures' in a blood film from a case of pernicious anemia in relapse. There is considerable variability in the appearance of cells in different portions of this film, and outside some of the cells fine granules occur either diffusely scattered or forming round or oval structures. Method A. × 1750.

Fig. 4 This preparation illustrates the effect of mild crenation upon the distribution of the granule substance. Note the pattern of the granule-formed architecture has been determined by the crenation. The granules have accumulated along the ridges and at the nodal points of the crenated surface. Other portions of the film show the more usual appearance. This phenomenon of the effect of crenation has been observed in numerous preparations and is considered strong evidence in favor of the artificial nature of the structure. Method A. × 1750.





Figures 5 and 6

nitely than others. Figure 7, from a blood film of the same patient, was treated by method A.

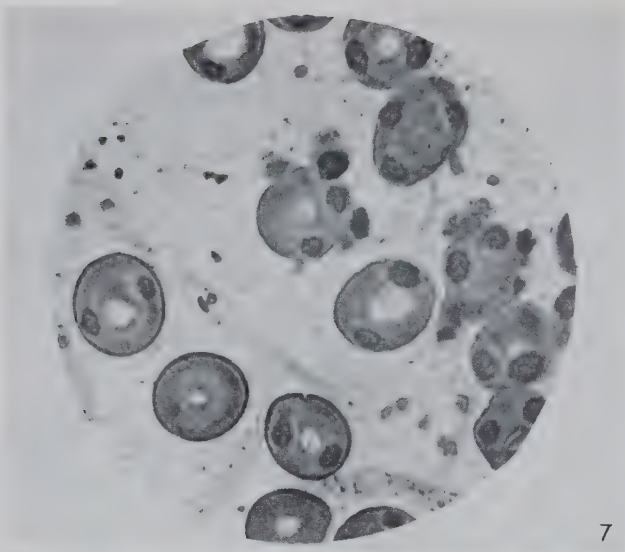
Figure 8 illustrates a variety of patterns in erythrocytes treated by the combined method. In some of the cells the granular substance forms densely stained structures at one or two poles of the cell. In other cells the zone of increased density occupies the center of the cell, recalling the reticulo-fibrillar apparatus of Golgi.

It would seem that the only sound interpretation that could be made of the observation is that the preliminary mordant of reduced molybdic acid and phosphotungstic acid, even in air-dried and methyl-alcohol-fixed films, occasions a shift and concentration of the hemoglobin into certain areas of the erythrocyte and that these zones of concentrated hemoglobin serve as a base for the precipitation of the sensitive silver solution. Zones of diminished hemoglobin concentration do not react. It is possible, too, that some of the hemoglobin is discharged from the cell.[3]

[3] Attention should be called to somewhat similar structural appearances produced in normal erythrocytes by Cooke(5) in which the homogeneity of the cell is disturbed by application of mixtures of alcoholic benzidine and hydrogen peroxide. It is considered by Cooke that the lipoid envelope is penetrated or dissolved and a reaction between the reagents named and hemoglobin takes place with solution and escape of the end products. It is known that the blue meri-quinonoid produce of the benzidine test for blood is dependent upon the oxidation of benzidine by hydrogen peroxide in the presence of hemoglobin which acts as a catalyst. Apparently depending upon the varying degrees of penetration of the lipoid envelope of the erythrocyte and the amount of hemoglobin released or reacted upon by the reagents, a variety of appearances are revealed; hemoglobin unreleased but reached by the reagents remaining variously as stippling and fili form or bar-shaped reticulation. Cooke believes the reaction is analogous to that of punctate basophilia, diffuse polychromasia, and reticulation revealed by the usual staining methods in certain erythrocytes, in such instances dependent upon abnormal penetrability of the lipoid envelope.

Fig. 5 A blood film treated by the combined method, but remaining but a short time in the phosphotungstic and reduced phosphomolybdic-acid bath. The granule substance (hemoglobin) has undergone but little dissociation or rearrangement. A few cells show an early formation of the oval structures. × 1750.

Fig. 6 Preparation of the same blood as illustrated in figure 5 treated by the combined method, but treated in the phosphotungstic and reduced phosphomolybdic-acid mixture for a much longer time. The 'structures' are more clearly seen. The cells illustrated show an apparent series of stages in the development of the structures. The different arrangement in the crenated cell should be noted. × 1750.



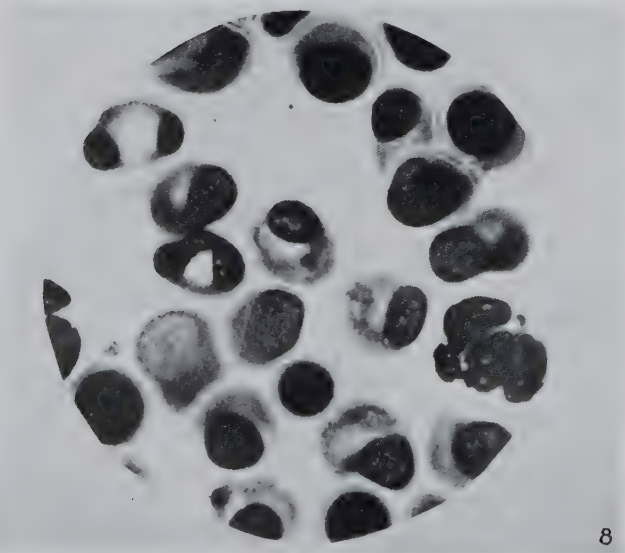


Fig. 7 Erythrocytes of same case as illustrated in figures 5 and 6 treated by method A. Note the accumulation of the granule material outside of the general contour of the cells in traumatized erythrocytes. × 1750.

Fig. 8 Erythrocytes treated by the combined method showing various forms of shift in the hemoglobin. Attention is called to the similarity in appearance between the reticulofibrillar apparatus of Golgi and the cells in which the hemoglobin has concentrated at the center of the cell. × 1750.

The unusual reaction here described would appear to bear some relation to previously described phenomena and 'structures' reported in the erythrocytes which are mentioned briefly in the following section.

THE MAXIMOW PHENOMENON

Wlassow(6), Maximow(7), and, more recently, Golgi(8), Gamma(9), Boccadoro(10), and Jordan, Kindred, and Beams(11) described an unusual reaction, generally known as the Maximow phenomenon, occurring in the erythrocyte when treated by special technical methods. The method of Gamma given below is easily performed and readily produces the reaction in question.

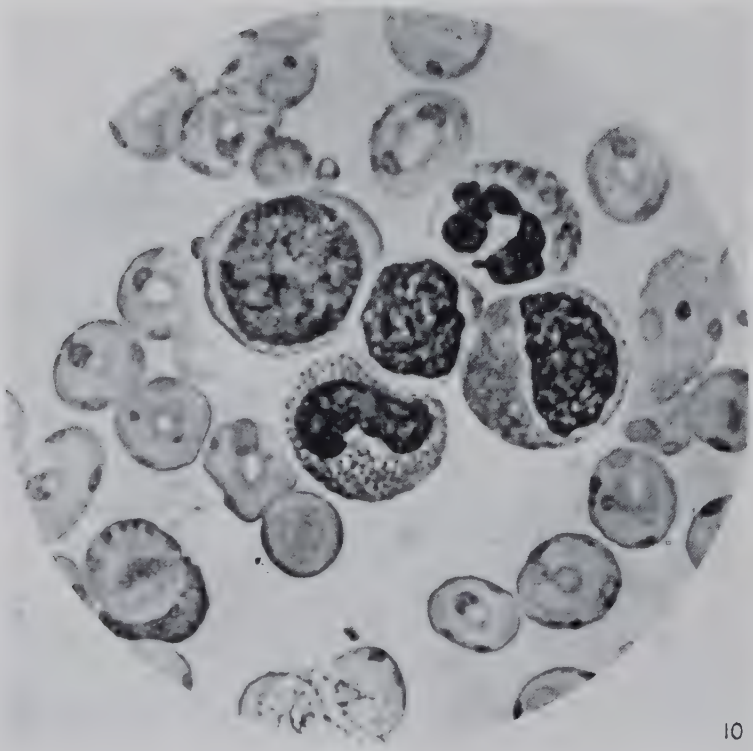
Method of Gamma

Wet blood films are treated with 75 per cent potassium-dichromate solution containing 0.5 per cent acetic acid and one or two drops of saturated solution of $CuSO_4$ for every 3 cc. of fluid. After from five to ten minutes, the films are washed in physiological salt solution, stained with eosin, washed and mounted in distilled water or dehydrated, cleared, and mounted in balsam.

Cells so treated show a single rounded projection extending from the surface of the erythrocyte. The knob-like projection and a portion of the cell beneath it appear homogeneous and stain with eosin or Bordeaux red. The interpretation of Jordan, Kindred, and Beams is that the hemoglobin had become dissociated and segregated into this zone.

It is considered probable by the author that the phenomena, illustrated in the accompanying figures, involve a dissociation and rearrangement of the hemoglobin similar, in general, to the Maximow reaction.

The author has applied the Gamma technique and superimposed his method of demonstration of hemoglobin. He agrees to the fact of the dissociation and rearrangement of the hemoglobin, but finds it to be somewhat different than



Figures 9 and 10

described by Jordan, Kindred, and Beams. The projection of the red blood cell is usually filled with hemoglobin. Adjacent to this projection is a narrow zone entirely free of hemoglobin (zona homogenea), while the area next to this (zona granulosa) contains hemoglobin. In the portion of the cell distal to the projection, hemoglobin is either absent or rarefied (fig. 9). This distribution corresponds rather closely to the distribution of the reticulofibrillar apparatus of Golgi in cells treated previously by the Gamma technique illustrated by Jordan, Kindred, and Beams.

COMMENT

Hematologic literature contains an extensive number of studies dealing with unusual structures or structural appearances in normal and pathologic erythrocytes.

Although the evidence perhaps is not complete that structures revealed by the technical method employed in this study are artificially produced, it would strongly favor the interpretation of a dissociation and rearrangement of the hemoglobin. If this is the correct interpretation, it would appear that many of the 'structures' described for the erythrocyte would bear closer scrutiny from this point of view.

Golgi(12) described a structure in the erythrocyte revealed by a special technique which has become known as reticulofibrillar apparatus. This appears as a centrally placed disc or ring-shaped granular or fibrillar zone usually coextensive with the normally pallid central area of the cell. Hartman (13) applied a modification of the technique and was led to believe that the substance stained was hemoglobin. It would seem probable that he was correct in this supposition, although it is quite obvious that it does not give an adequate or true picture of the hemoglobin. The author considers that

Fig. 9 Normal human erythrocytes treated first by the method of Gamma and subsequently by the author's method for impregnation of hemoglobin. × 1750.

Fig. 10 The blood of chronic myelogenous leukemia. Note the precise delineation of the nuclei of a group of cells including a myeloblast, two neutrophilic myelocytes, an eosinophile, and a neutrophilic polymorphonuclear leucocyte. Method A. × 1750.

this apparatus may be another form of dissociation and concentration of the hemoglobin. Jordan, Kindred, and Beams noted an intimate relation between the hemoglobin and the reticulofibrillar apparatus of Golgi and described the coextensive occurrence of this apparatus and the hemoglobiniferous zone in cells modified by the Gamma technique. The author has observed, in some of his preparations made by the combined method, structures bearing a striking resemblance to the reticulofibrillar apparatus of Golgi (fig. 8).

Schilling-Torgau(14) reviewed the literature and presented his own observations on the wide variety of unusual staining reactions encountered in the erythrocyte and built up an elaborate theoretic composite architecture for this cell, including 'structures' described as Glaskörper, Innenkörper, Kapselkörper, Pseudonucleode, Blutplättchen, etc. Most methods used in the demonstration of these architectures employed procedures involving some degree of hemolysis. It was considered that hemoglobin cleared the cell, revealing these internal structures. In light of the present study it would seem probable that some of these structures represent artificial architectural appearances due to internal rearrangement and partial discharge of hemoglobin. Although the structures produced by the technique of the author are probable artificial, it is not improbable that with a sufficiently well-controlled technique, a fairly constant pattern might be found in various pathologic states of the erythrocytes.

VALUE AS NUCLEAR STAIN

Quite aside from the question of the nature of the erythrocytic structures, the technical method employed (method A) has been found to give unusually precise detail of nuclear architecture. This alone would appear to make the method of value. Figure 10 shows the precise delineation of the chromatin in a group of white blood cells from a case of chronic myelogenous leukemia. The method is of little value in study of the white blood cell granules. Azurophilic or neutrophilic granules do not stain. Eosinophilic granules give a translucent yellow reaction and basophilic granules stain black.

SUMMARY AND CONCLUSIONS

Unusual 'structures' are described in the erythrocyte revealed by a special technical method which is given in detail. Evidence is presented indicating that the phenomenon probably is artificially produced by an intracellular dissociation and rearrangement of the hemoglobin. The relation of this to the Maximow phenomenon and to previously described structures in the erythrocyte is discussed.

Attention is called to the precise staining of nuclei of the white blood cells secured in employment of the method.

The author wishes to express his appreciation to Miss Adelaide Hayes for expert assistance in preparation of microphotographs illustrating this study.

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A MOVING-PICTURE APPARATUS FOR MICROSCOPIC WORK

BRADLEY M. PATTEN AND THEODORE C. KRAMER

Laboratory of Histology and Embryology, Western Reserve University School of Medicine, and The Baldwin Research Laboratory,[1] *Cleveland, Ohio*

SEVEN FIGURES

Since the appearance of the pioneering work of Comandon ('09) biologists have been increasingly interested in moving pictures as an aid to teaching and investigation. From the teaching standpoint, the permanent recording of precarious experiments on living material in a manner which makes it possible to demonstrate them with assurance to a group of any size at any desired time is in itself of the greatest value. The gradual accumulation of such records, each executed by a specialist in some particular field, and the development of an adequate interuniversity exchange system which could follow as a natural sequel would offer a vividness and variety of teaching material such as no single institution could otherwise command.

To the investigator the way in which the motion-picture camera affords a mastery of the time element opens new vistas. Growth changes too slow to be fully appreciated even by the continuous day and night observation of investigators working in relays, yield the details of their mechanism to a motor-driven camera. For a film taken at the rate of one exposure per minute, when viewed at the normal projection rate of sixteen frames a second, accelerates the action 960 times. If that is too fast or too slow, adjustment of the timing mechanism is all that is needed to reproduce the process at the optimum rate for critical study. As any frequenter of the 'movies' is well aware, equally interesting possibilities exist in the analysis of actions too rapid for the eye to follow.

[1] Contribution no. 22 from The Baldwin Bird Research Laboratory.

The fact that as yet relatively few people are working with the method is largely responsible for the impossibility of purchasing an adequate apparatus assembled and ready for use. It is true that there are available portable outfits, operating with 16-mm. film, which are capable of yielding satisfactory results if the photographic problem is not too exacting. Within their limitations such outfits are of great value. There seems, however, to be no question in the mind of anyone who has attempted to do much work with moving pictures of microscopic objects that, for anything other than the simplest of subjects, photographed at moderate magnifications, the use of standard 35-mm. negative is indicated. Once successfully assembled and edited, a standard negative can readily be printed down to 16-mm. positives for economy in distribution. Moreover, standard size positives—the only ones suitable for projection in large rooms—can always be made from such a master negative, whereas the 16-mm. negative cannot be 'stepped up' to standard size without serious loss of detail.

Working independently in highly specialized fields, Canti, Lewis, Moore, and Bayne-Jones have developed apparatuses capable of yielding superlatively fine results with standard film. All these outfits are, however, built primarily for 'time-lapse' work with transparent material, as is the elaborate, and for most institutions, prohibitively expensive apparatus described by Rosenberger ('29). As far as we were able to ascertain at the time we became interested in this type of work no one had developed a relatively simple apparatus sufficiently flexible in construction to permit its use under a wide range of conditions. Any work with micro-moving pictures is expensive at best, and institutions that can afford to maintain a series of such apparatuses, each built for a special type of work, are at present virtually non-existent. The problem, therefore, of developing an apparatus capable of doing the wide variety of work which might be demanded of it by the staff of an active biology department was an interesting challenge. The undertaking of work toward this end

was made possible through the unflagging enthusiasm and generous financial support of Mr. S. Prentiss Baldwin, of Cleveland, founder and director of the Baldwin Bird Research Laboratory.

No useful purpose would be served by describing the anatomy of the five evolutionary forebears of our apparatus as it now stands. Suffice it to say that each successive change in design carried us further away from the original arrangement with all its parts mounted on a common support; and that increased weight of independent bases and ever more rigid supports for moving parts has been the general trend of the changes. Nor are we suffering under any delusions that in its brief series of new adaptations our apparatus has evolved into anything like perfection. Its basic design has, however, proved practical for widely varying types of work. It has yielded satisfactory pictures taken at rates varying from one exposure in two minutes to forty exposures a second. It can be used for photographing single cells or small cell groups with objectives as high as 4 mm. dry or the lower-powered oil immersions. At the other extreme, with a 100-mm. lens, it can take pictures of such objects as hatching wren embryos at an initial magnification on the negative of only two or three diameters. Its independently movable lighting unit makes possible the use of transmitted light for photographing transparent objects at high magnification, or reflected light for photographing opaque objects at low magnification. No such 'all-purpose' apparatus can be as refined an instrument for specialized work as, for example, that constructed by Bayne-Jones for the exceedingly high magnifications necessary in dealing with bacteria, or that set up by Moore and Pillsbury with tandem microscopes for an increased depth of focus, or that now being developed by Chambers and his associates for the recording of microdissection and micro-injection experiments. But as equipment meeting the general demands which might be made on it as a departmental installation, a less highly specialized and more flexible apparatus has distinct advantages. For this reason,

and also because the simplicity of its construction makes possible the assembling of the apparatus at a relatively moderate cost (table 1), we are publishing the following description of the apparatus with which we have been working. While the dimensions are available from the scale diagrams, the description in the text has been kept to general terms, because we feel that anyone building such an apparatus would follow only the general design and modify or improve details to suit his own purposes.

TABLE 1

Approximate outlay necessary for constructing the apparatus, assuming that a microscope properly equipped for photographic work is available

Camera (cost second-hand, much variation)	$150.00
Cold-rolled 1¼-inch steel rods for tracks	7.50
One pattern, two castings, for sliding carriages	18.00
Cement, sand, and stone for bases	2.50
Strap iron for spacers and braces	3.50
Scrap lead for counter weights	3.50
Motor	15.00
Rheostat for control of motor speeds	10.00
Reduction gear assemblies	35.00
Automatic arc with collecting lenses[1]	65.00
Incandescent microscope lamp[1]	25.00
Materials for making frame for carrying lights	10.00
Small electric incubator for microscope[1]	50.00
Modified demonstration ocular beam divider	70.00
Miscellaneous small hardware and findings	15.00
Shop work. Assuming there is in the department a shop with fair facilities where all the simpler work will be done, allow for machine work sent out	100.00
Total	$580.00

[1] Need not be of any specific type. Any serviceable one available can be utilized.

The general plan of the apparatus is shown by figure 1. It consists of four main parts: 1) the microscope-incubator assembly mounted on vertical steel rods set in a 500-pound concrete base; 2) the camera assembly, also mounted on vertical rods, parallel to the microscope track, and set in a second, independent, 500-pound concrete base; 3) the lighting assembly, consisting of a movable stand carrying an arc light, and

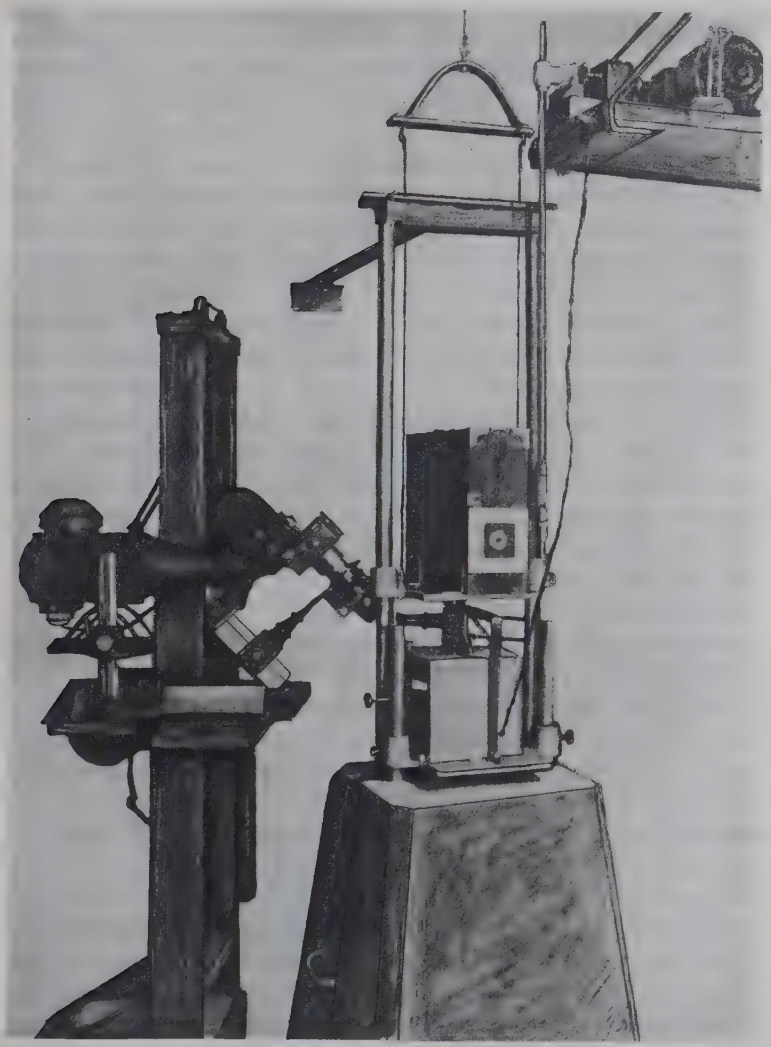

Fig. 1 General view of apparatus to show the interrelations of its various parts. For details of the several units see figures 2 to 6. Note especially the position of the arc lamp, which is set for photographing by reflected light.

a 400-watt concentrated filament Mazda lamp, both provided with suitable housings and collecting lenses; 4) the driving and timing assembly, consisting of a motor and appropriate reduction gears mounted on a shelf on the side wall of the room.

The most important feature of this plan is the complete separation of the camera and the microscope. There is a certain amount of vibration unavoidably associated with the 'jump-ahead-and-stop' mechanism carrying the film through a moving-picture camera. At the slow camera speeds used in time-lapse photography of growth processes this vibration is negligible. But when the film is run sufficiently rapidly to reproduce actions at their normal rate (i.e., sixteen to twenty exposures a second), this vibration becomes a serious problem. If the camera mechanism is good, the amplitude of the vibration is so small that it does not interfere in any way with the efficiency of the camera. If, however, this same vibration is imparted to the microscope and thereby magnified, blurred images result. We at first believed that a 1000-pound base for the camera track would offer sufficient inertia so that the microscope could be set on it without being affected by vibration. With low magnifications and normal camera speeds this was a workable arrangement. With high magnifications or with camera speeds around thirty to forty exposures a second there was sufficient vibration transmitted from the camera to the supporting rods, and thence to the cement base and the microscope, to cause some blurring of the image. The use of separate bases for camera and microscope entirely obviated this difficulty.

The reasons for mounting the driving and timing mechanism independently of the rest of the apparatus are obvious. The only connection is the driveshaft to the camera, which is rubber-coupled to avoid the transmission of any vibration from motor to camera.

The use of an independently movable lighting unit was dictated by several considerations. In the first place, manipulation of the lights could be carried out without risk of

jarring any other part of the apparatus. Secondly, delicate variations in intensity could be secured by altering the distance of the source of the light from the object. Thirdly, it offered the possibility of readily interchanging the intense light of an arc, necessary for photographing at high speeds, with a less intense light suitable for making interval exposures. Finally, it offered the possibility of using oblique reflected illumination for photographing older, opaque embryos which cannot be photographed successfully by the use of transmitted light. (Note position of arc in fig. 1.) The flexibility of the apparatus as a whole depends more upon this movable lighting unit than upon any other feature of its construction.

Most of the essentials of the construction of the various units are shown in the scale drawings of figures 2 to 6. For those interested in the possibility of building a similar apparatus, some of the details of the construction of the several units are appended to the foregoing general description.

BASES AND OPTICAL TRACKS

The concrete bases were poured in home-made forms in the laboratory where they were to be set up. The height of the top of the bases above the floor was determined simply by convenience of working height for the microscope. In the forms, before the cement was poured, were set the 1¼-inch cold-rolled steel rods that were to serve as the optical tracks. These were held in place by spacers made out of ⅜-inch × 2-inch strap iron which had been clamped together and simultaneously bored to insure the parallel setting of the two rods. For the microscope-incubator base the rods should project above the concrete about 60 cm. to permit carrying the long-focus, low-power lenses. (See section on microscope.) For the similar base carrying the track for the camera, the rods should extend at least 120 cm. above the top of the concrete base (fig. 2). Liberal use of spacers and frequent checking with a level during the pouring of the cement are necessary to insure the parallel setting of the rods.

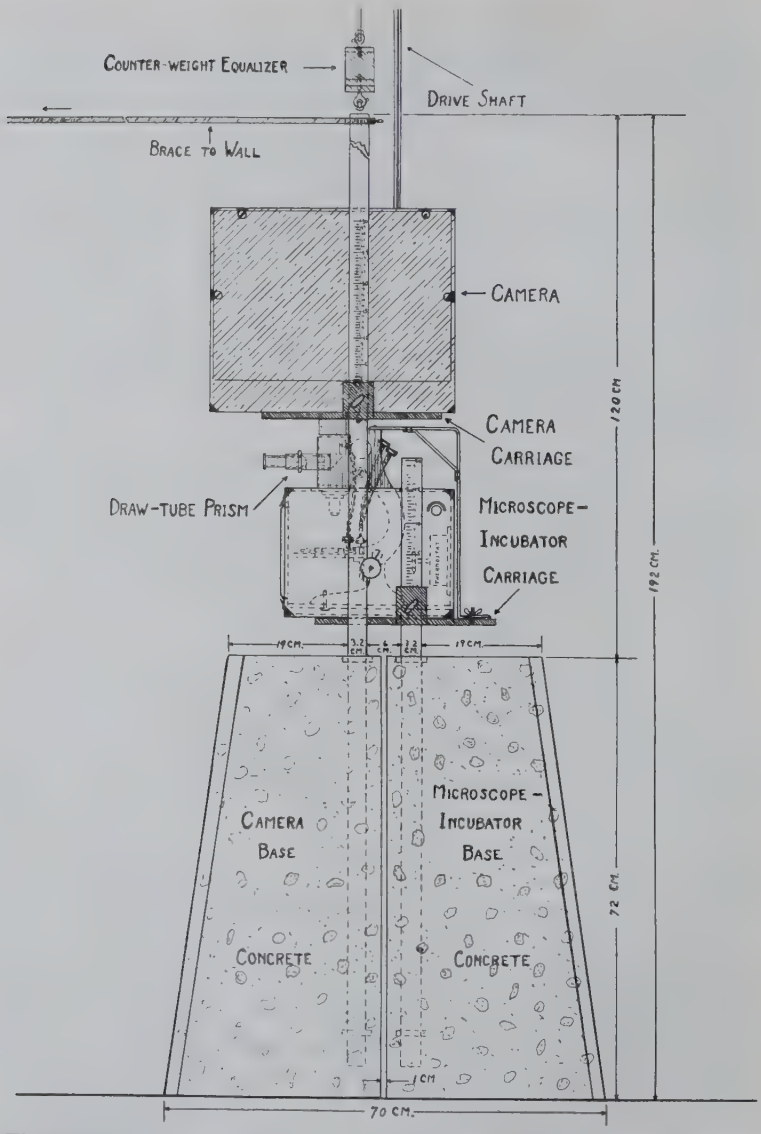

Fig. 2 Scale plan of apparatus in side elevation. The pulleys for the counterweight are attached to the ceiling and therefore out of the field of the drawing. The break in the upper part of the optical track indicates a section omitted for bringing the drawing into more compact form. Compare with figures 1 and 3.

Aluminum castings were made to act as carriages sliding on these two parallel tracks. To secure smooth motion of these carriages on the track the holes were 'bushed' with bronze. Set-screws permit the locking of the carriages at any desired height. The carriages must be of sufficiently generous size so that the lens aperture of the camera and the optical axis of the microscope can be brought into the same line (fig. 2).

THE INCUBATOR

A small electric incubator like the type 10 for sale by the Chicago Surgical and Electrical Company encloses the microscope. The simple construction of this incubator permitted its alteration to accommodate certain additions to the microscope accessories that we found it advisable to make. The substitution of a glass plate for one of the top slides of the incubator permits oblique illumination from above the stage. The incubator as manufactured carries a glass plate in front, which permits the illumination of the object by transmitted light from the microscope mirror.

The microscope was fixed in the incubator by means of three $\frac{3}{16}$-inch brass pins set with lock-nuts into the incubator floor and fitting into holes drilled in the feet of the microscope base. To facilitate its proper centration, the microscope-incubator assembly was not rigidly bolted to its carriage. Metal blocks were affixed to the floor of the carriage, so that there was about $\frac{1}{4}$-inch space between the blocks and the base of the incubator placed approximately in its correct position. Set-screws in these blocks give the necessary play for accurate centration under the camera. When the centering has been accomplished, tightening the set-screws locks the incubator in place. To further insure rigidity of the entire assembly a right-angled clamp was made of strap iron and so fitted that when one end was bolted to the carriage, the other end pressed down on the top of the microscope pillar (fig. 2).

MICROSCOPE

The standard type of microscope needs some modification and the addition of special accessories for use with a moving-picture apparatus. The changes we found necessary were sufficiently simple to permit of their being accomplished in our own shop, and the accessories are, for the most part, available in the market. A large photomicrographic barrel with a side-view tube, carrying at the end of a movable sleeve a prism that can be brought into the optical axis or completely withdrawn at will, was substituted for the standard barrel. A barrel so equipped is made by E. Leitz for their large photomicrographic apparatus. By substituting for the prism a cover-glass set at an angle of 45°, the major part of the light is transmitted to the camera, while sufficient light is reflected into the side tube to permit following the action at the same time that it is being photographed. This device is employed with 32-mm. to 100-mm. objectives, which work more satisfactorily without the use of an ocular. For the higher-powered objectives with which it is desirable to use an ocular a modified demonstration ocular assembly is used, which divides the light so that upward of 90 per cent goes through to the film, while 10 per cent or less is reflected into the side tube for observation of the material being photographed. Several of the microscope manufacturers are now making this assembly especially for photographic work.

The microscope was next equipped with a focusing stage. In making this change the stage was freed from its normal attachment and affixed to the rack and pinion provided for the condenser. The condensor was then reset by using one of the old-type condenser carriers attached to an underneath corner of the stage. Microscopes constructed with a focusing stage can be bought and would be preferable to this adaptation which we found it expedient to use.

Manipulation of the object was secured by means of an ordinary mechanical stage, the controls of which were extended by affixing to them pieces of speedometer cable with milled-heads attached to the ends which projected outside the

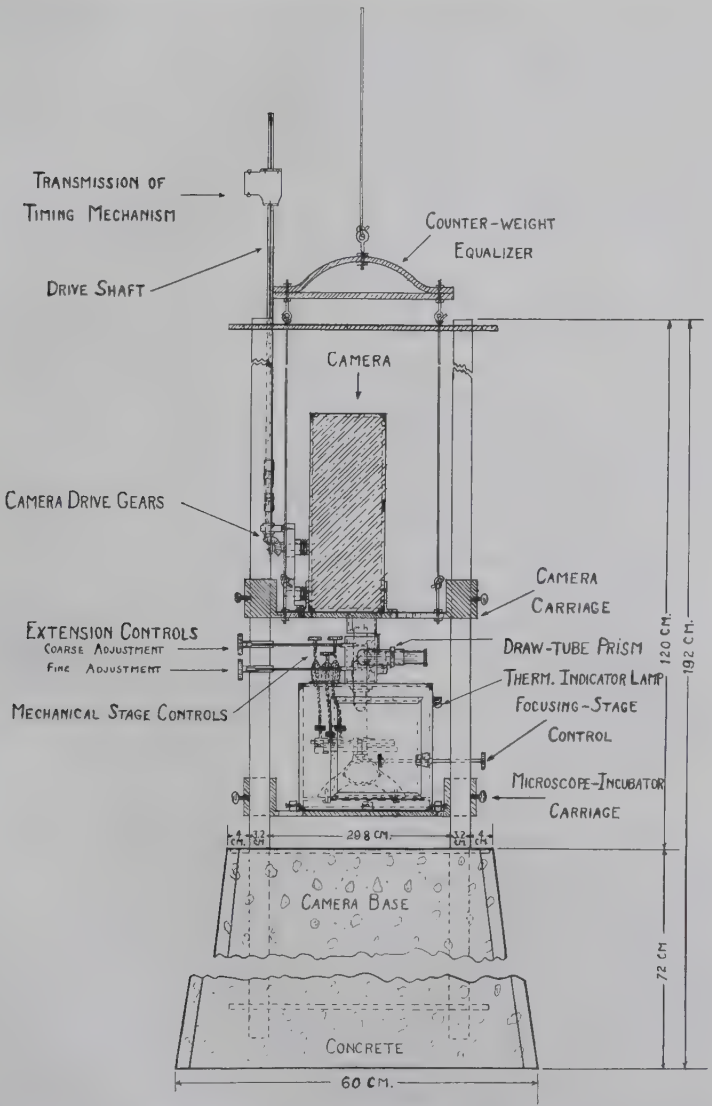

Fig. 3 Scale plan of apparatus in front elevation. The breaks in the base and in the optical tracks indicate sections omitted for convenience in drawing.

incubator (fig. 3). Rotation of the object was obtained by fixing in the jaws of the mechanical stage a brass plate into the center of which a thin gear wheel was set. This gear could be rotated by a small peripherally meshed cogwheel which was also controlled from outside the incubator by a speedometer cable extension. This makeshift adaptation, though fairly efficient, could and should be obviated by starting with a microscope equipped with a rotating mechanical stage.

The milled-heads controlling the coarse and fine adjustments of the microscope were removed from one side and carried out on flexible extension cables to avoid the necessity of working in the cramped space between the top of the incubator and the bottom of the camera carriage, and a similar extension control was affixed to the focusing stage (fig. 3).

To carry the low-power, long-focus objectives a sliding frame was constructed to operate on the microscope-incubator track. This frame had a slot fitted to the rack of the microscope barrel so that the barrel could be carried to a greater height above the stage than is permitted by the rack and pinion of the microscope pillar. Centration of this assembly so that the microscope barrel is carried above the microscope pillar in proper alignment was a simple matter of trial and error and shims.

CAMERA

The camera which we finally incorporated in the apparatus was an old-style box camera. Ours was a Moy camera, but there are several other makes that would serve as well. Because box cameras have now been replaced commercially by more compact aluminum-cased instruments, they can be procured second-hand at very low prices. Their mechanism is excellent and their size is no handicap when they are to be built into a fixed piece of apparatus. One advantage of such cameras for this type of work is their possession of two driveshafts, one geared so that it makes one exposure for each revolution of the crank, and the other geared so that it makes

eight exposures for each crank revolution. A second advantage is the focusing tube which permits one to look through a telescopic device in the top of the camera at the image on the film enlarged by a magnifying glass. This mechanism for focusing directly on the film is of the greatest advantage in microscopic work.

The entire camera-carriage-assembly is counterweighted (fig. 3) so that it moves smoothly and readily on the optical track. A scale was cut in one of the members of the track which permitted the recording of the proper camera setting for various lens combinations.

THE DRIVING MECHANISM OF THE CAMERA

The entire driving and timing mechanism is mounted on a shelf on the wall above the apparatus and connected to it only by a rubber-coupled driveshaft. The arrangement of this unit of the apparatus is shown in figure 4. A $\frac{1}{6}$-horsepower, 2450-R.P.M., direct-current motor furnishes the driving force. The advantage of the D.C. motor is the possibility of precisely controlling its speed through the use of a large rheostat. To bring the speed of the motor to the order of magnitude necessary for operating the camera, two speed-reducing units, bought from the Boston Gear Company, were used. These reducers are set up so that the drive can go direct from a 10-to-1 reducer to the driveshaft transmission, or, by sliding a gear wheel into mesh, a 100-to-1 reducer can be brought into the circuit. (See shift gear in fig. 4.) For the normal speed and ultrarapid work the 10-to-1 reducer alone is used and the drive is coupled to the 8-to-1 camera crankhead. For pictures made at intervals of thirty to sixty seconds the 100-to-1 reducer is thrown in and the drive shifted to the 1-to-1 camera crankhead. The rheostat control of the motor gives the necessary flexibility for arriving at the exact speed desired.

From the driving and timing mechanism the power is transmitted through a beveled gear to a vertical driveshaft. The cutting of a long key-way in this shaft permits it to be

engaged in the transmission and still allows the raising and lowering of the camera without any adjustments of the driving mechanism. A second set of beveled gears transmits the

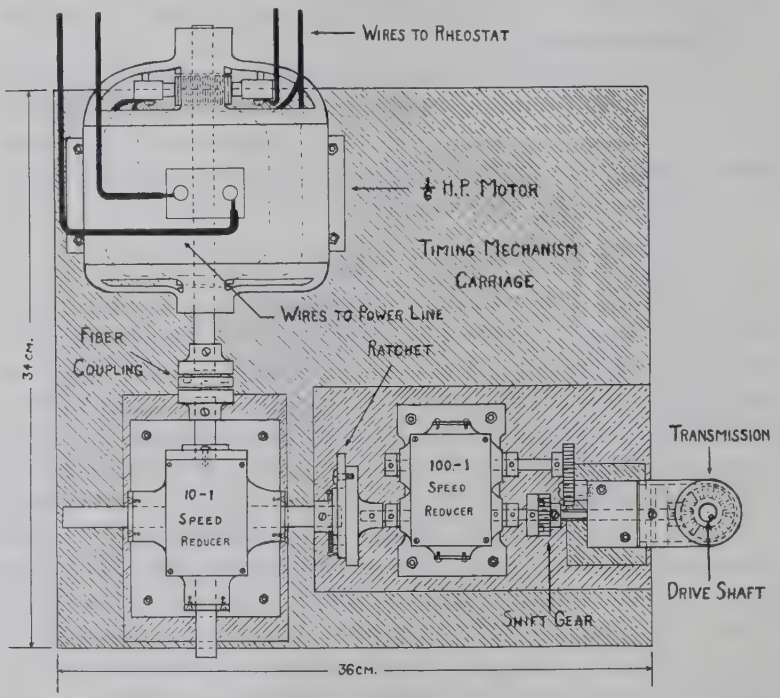

Fig. 4 Scale plan of arrangement of motor, speed-reducing gears, and transmission. This entire assembly is mounted on an independent shelf on the side wall of the room. The ratchet between the 10-to-1 and the 100-to-1 speed reducers provides for connections with an electromagnetic timing device for making exposures at intervals greater than one per minute. This feature of the mechanism was not described in the text, because it has not yet been used in actual work.

power from the driveshaft to the camera crankheads (fig. 3). The block which carries these gears has two 'bushed' openings opposite the two crankheads in the camera case, thus permitting the shifting of the gears to operate either camera crankhead.

LIGHTING ASSEMBLY

The basis of the illuminating mechanism was a small portable Leitz microprojection apparatus. The optical track of this apparatus was cut in half. On one half of the track was mounted its clockwork arc light and collecting lenses. On the other half of the track was mounted a Spencer incandescent microscope lamp (catalogue no. 394). The base which formerly carried the microscope of the dismembered projection apparatus was used for carrying the Spencer lamp (fig. 5). The tracks for these two lights were mounted parallel to each other on a platform which could be raised to the desired height on a frame constructed of 2×4-inch wooden uprights. These uprights were supported on a base provided with casters and weighted with concrete. The movable platform and all the accessories which it carried were counterweighted to facilitate raising and lowering the assembly to the desired height. A front elevation of the apparatus is shown in figure 5 and a plan of the layout on the platform in figure 6.

The track carrying the arc lamp was mounted in such a manner that it could be swung through an angle vertically or horizontally. Control of direction in the horizontal plane was obtained by pivoting the end of the track toward the microscope and cutting the back part of the supporting platform on the arc of a circle of which the track was the radius (fig. 6). A set-screw locks the rear end of the track at any desired point on this arc. Change in the inclination of the light was provided for by hinging the optical track at its front end and attaching a slotted supporting arm at the rear end. A set-screw engaging on the supporting arm permits the fixing of the optical track at the desired angle (fig. 1).

Very little comment on the use of such an apparatus will be needed by anyone sufficiently interested in this type of work to have studied through the details of the construction. Once the apparatus has been assembled, most of the technical problems encountered will center about the matters of illumination and exposure times. The following examples of photographs made for different purposes and under quite dif-

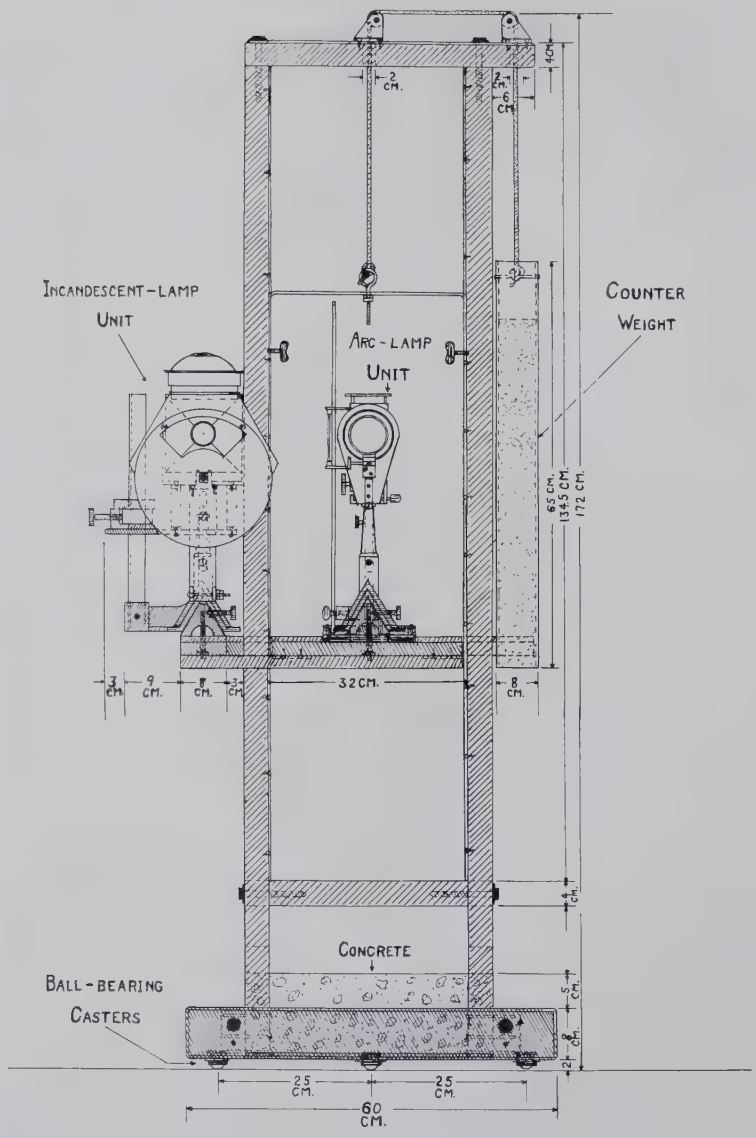

Fig. 5 Scale plan of lighting unit in front elevation. For detailed plan of platform carrying the lights see figure 6.

ferent conditions may serve to reduce the inevitable wastage in standardizing photographic conditions for a new apparatus.

By setting the supporting platform of the lighting unit so that the arc lamp is opposite the microscope mirror, intense illumination suitable for taking normal speed pictures of transparent material is obtained. Figure 7, A, is a print of a sample frame of film made from a living chick embryo of

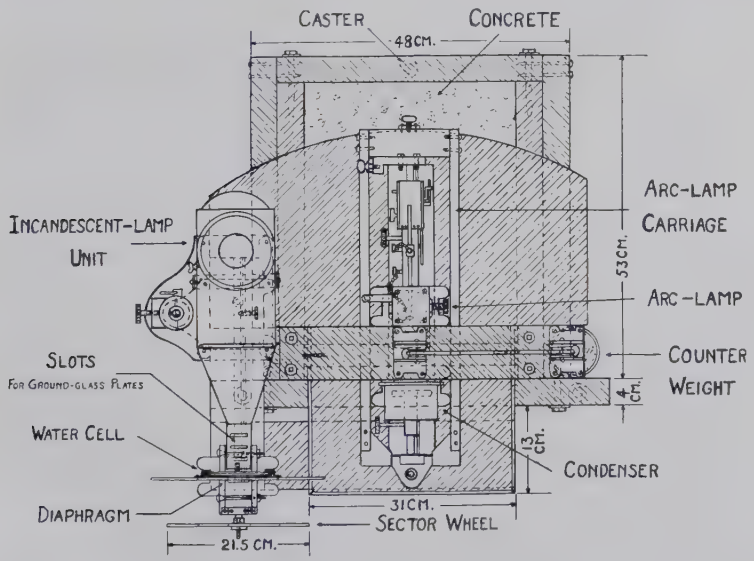

Fig. 6 Scale plan showing the arrangement of the lights on the adjustable platform.

thirty-four hours' incubation to reproduce the heart action on the screen at its actual rate. That is, the pictures were taken at sixteen frames a second for projection at the same rate. Figure 7, B, is from a film taken at normal speed, but at considerably lower magnification, so that the field would include some of the yolk-sac vessels to show the movement of blood as well as the heart action. Figure 7, C, was also taken at normal speed, but at a higher magnification, so that the movement of the corpuscles in the vessels might be followed.

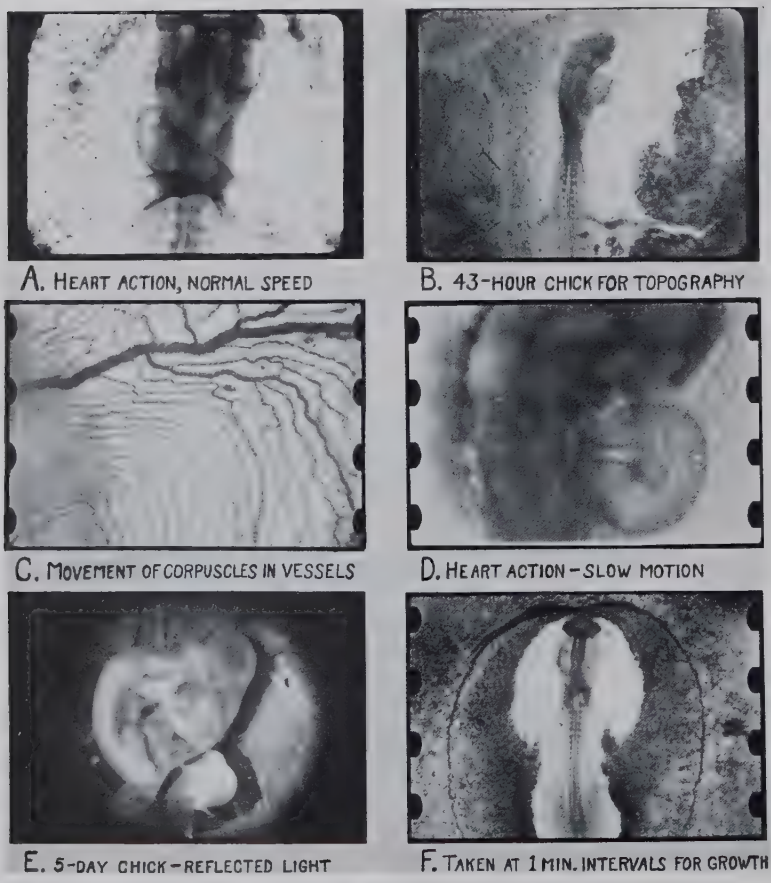
A. HEART ACTION, NORMAL SPEED
B. 43-HOUR CHICK FOR TOPOGRAPHY
C. MOVEMENT OF CORPUSCLES IN VESSELS
D. HEART ACTION - SLOW MOTION
E. 5-DAY CHICK - REFLECTED LIGHT
F. TAKEN AT 1 MIN. INTERVALS FOR GROWTH

Fig. 7 Prints from single frames of film, illustrating results from different kinds of subjects taken under various conditions as to illumination, magnification, and timing.

A. Heart of thirty-four-hour chick taken to show beat on screen at its actual rate; 16-mm. objective; no ocular; transmitted light from arc distant 18 inches from microscope mirror; sixteen exposures a second; shutter aperture, 90°.

B. Forty-three-hour chick and adjacent vascular area, taken to show circulation as a whole at normal speed; 42-mm., F 4.5, micro-summar objective with diaphragm at 6; no ocular; transmitted light from arc distant 20 inches from mirror; sixteen exposures a second; shutter aperture, 90°.

C. Part of the vitelline vascular plexus of a seventy-two-hour chick taken to show movement of blood corpuscles at their actual rate; 32-mm. micro-tessar objective with diaphragm at 6; no ocular; transmitted light from arc 18 inches from mirror; sixteen exposures a second; shutter aperture, 90°.

Details as to lenses and exposure time for each example are given in the figure legend.

Ultrarapid pictures are taken with the same arrangement of the apparatus except that the arc lamp is moved closer, and the shutter aperture in the camera is increased to compensate for the very short exposures demanded. Figure 7, D, is a print from film made from the heart of a forty-four-hour chick at the rate of forty exposures a second. When projected at normal speed, the heart action appears sufficiently slowed to permit analysis of the nature and progression of the contractions.

By wheeling the entire lighting unit around to the side of the microscope base, raising the platform, and inclining the optical track downward (fig. 1), an opaque embryo can be illuminated diagonally from above by a light sufficiently intense to permit photographing it by reflected illumination. Figure 7, E, shows a picture of a five-day chick made in this manner. The value of having the side-view mechanism in the microscope constructed as a sliding sleeve should, perhaps, be emphasized in connection with pictures taken thus by reflected light. Because only a relatively small proportion of the light used is reflected from the object to the film, it is frequently exceedingly difficult to secure adequate illumina-

D. Heart of a forty-four-hour chick taken at high speed for slow-motion projection; 16-mm. objective; no ocular; transmitted light from arc 10 inches from mirror; forty exposures a second; shutter aperture, 120°.

E. Five-day chick taken to show general form of embryonic body; 64-mm. F 4.5 micro-summar objective with diaphragm at 6; no ocular; reflected light from arc 10 inches from embryo, light passed through a 5-cm. water cell; sixteen exposures a second; shutter aperture, 90°.

F. Entire chick cultured in chemical medium in glass chamber and photographed at one-minute intervals to show on screen growth changes accelerated 960 times; 42-mm., F 4.5 micro-summar objective with diaphragm at 8; no ocular; transmitted light from incandescent microlamp at 11 inches from mirror; three ground glasses and water cell interposed in beam; one exposure each minute; shutter aperture, 70°.

In using a camera with a rotating circular shutter having an adjustable aperture, the exposure on each frame of the film is:

$$\frac{\text{Angle of shutter aperture in degrees}}{\text{Total circumference of shutter (i.e., 360°)}} \times \frac{1}{\text{exposures per second}}.$$

For example, the frame exposure in A would be $^{90}/_{360} \times {}^{1}/_{16} = {}^{1}/_{64}$ second.

tion for taking pictures as fast as sixteen a second by reflected light. Even the slight ($\pm$ 15 per cent) reduction of light entailed by the side-view mechanism sometimes makes the difference between poor and good results. When the same embryo is not being followed for any great length of time, the side-view device is not necessary and the possibility of entirely withdrawing it from the path of light to the camera is most helpful.

For taking pictures at intervals which permit of relatively long exposures the steady illumination of the incandescent lamp is preferable to the arc. Control of the intensity of light suitable for varying lengths of exposure and various types of material can readily be obtained by altering the distance of the light from the mirror, and further, by the insertion of one or more ground-glass plates in slots at the end of the metal housing of the lamp. Figure 7, F, is a print from one frame of a film taken at minute intervals during the period of the bending of the cardiac tube. When projected sixteen frames a second, growth processes are sufficiently accelerated to be seen on the screen as active changes.

When it is planned to follow a living embryo for some length of time, taking pictures at, say, one-minute intervals, the side-view tube and the focusing stage come into play. The object is first focused on the film and then, by adjusting the ocular in the sleeve, it is brought into coincident focus in the side tube. The sharpness of the image can then be checked at any time and, by using the adjustable stage to keep the object in focus in the side tube, it is automatically kept in focus on the film.

If one desires to take, at intervals, slow exposures by reflected light, the entire incandescent lamp unit can be interchanged with the arc lamp to take advantage of the hinged and pivoted track described for the arc lamp.

When working for long periods of time with material which suffers from exposure to intense illumination, a sector wheel, mounted on a small saddle-stand fitting the tracks carrying the lights, can be placed in front of either lighting

unit. The sector wheel is then connected by flexible cable with the camera driveshaft and so synchronized with it that the object is illuminated only when the shutter of the camera is open for an exposure. With this precaution and the use of water cells to cut out as much heat as possible, the damaging effect of intense illumination can be held fairly well under control.

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